Investigation of Cis and Trans-acting Transcriptional Regulatory Factors and Signaling Pathways of *Parkin*

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Abstract

Parkin gene is associated with the development of autosomal recessive juvenile parkinsonism (Kitada et al., 1998) which is a common form of familial Parkinson's disease (Klein and Schlossmacher, 2006). Since Parkin has multiple cell protective effects, increasing the expression level of Parkin in the brain might be able to rescue cells in danger, which in turn might prevent or slow down the development of Parkinson's disease (Ulusoy and Kirik, 2008).

In order to increase Parkin expression, it is important to understand the transcriptional mechanisms regulating *Parkin* expression (Maston et al., 2006). Since human *Parkin* is very big (~1.4 Mb) (Asakawa et al., 2001), in this study we use the smaller Fugu *parkin* gene, which is an ortholog of human *Parkin* (Yu et al., 2005), to search for the transcriptional factors and signaling pathways regulating *Parkin* expression. We have cloned vertebrate constructs that allow for the monitoring of an entire genomic Fugu *parkin* gene tagged with a reporter (eGFP or luciferase) in mammalian cells; and have established cellular model for studying the expression.

According to the "*TRANSFAC*" transcription factor database, as well as "*TFBIND*" and "*TFSEARCH*" softwares (Wingender et al., 1996; Heinemeyer et al., 1998; Heinemeyer et al., 1999; Tsunoda and Takagi, 1999; Akiyama 1995), potential Nrf2 binding sites are conserved in the promoters of mammalian *parkin* (including human *Parkin* and mouse *parkin*) and in Fugu *parkin*. In this study, we could not find a link between the presence of the potential Nrf2 binding site(s) in the *parkin* promoter and the up-regulation of *parkin*; and we could not find an association between the Nrf2 pathway activation and the induction of parkin under the specific experimental conditions.

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List of abbreviations

Abbreviation	Full name
ACO2	Aconitase
ANOVA	Analysis of variance
ARE	Antioxidant responsive element
AR-JP	Autosomal recessive juvenile parkinsonism
ATF4	Activating transcription factor 4
BAC	Bacterial artificial chromosome
BCA	Bicinchoninic acid assay
bp	Base pair
BSO	L-Buthionine-sulfoximine
BTB	Broad-Complex, Tramtrack, and Bric àbrac
bZIP	basic leucine zipper
CBP	CREB binding protein
cDNA	Complementary DNA
CHD	Chromo-ATPase/helicase DNA binding protein
ChIP	Chromatin immunoprecipitation
CHO	Chinese hamster ovary
CREB	cAMP responsive element binding protein
CMV	Cytomegalovirus
CNC	Cap'n'Collar
CO_2	Carbon dioxide
COMT	Catechol-*O*-methyltransferase
C-terminus	Carboxyl-terminus

$CuCl_2$	Copper II chloride
Cul3	Culllin 3
DA	Dopamine
DEM	Diethyl maleate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
EGFP	Enhanced green fluorescent protein
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
GST	Glutathione S-transferase
GBA	β-glucosidase
GRP78	Glucose-regulated protein 78
Tris-HCL	Tris-hydrochloride
HEK	Human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HNE	4-Hydroxy-2-trans-nonenal
H_2O_2	Hydrogen peroxide
HO-1	Heme oxygenase-1
HRP	Horseradish peroxidase

IBR	In between RING-finger
IVR	Intervening region
Kb	Kilo-base
kDa	Kilodaltons
Keap1	Kelch-like ECH-associated protein1
L-DOPA	Levodopa
LRRK2	Leucine-rich repeat kinase 2
MAO	Monoamine oxidase
MAO-B	Monoamine oxidase B
Mb	Megabase
MBP	Maltose-binding-protein
MPP^+	1-Methyl-4-phenylpyridinium
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger ribonucleic acid
Mrps	Multidrug resistance-associated proteins
mtCK	Mitochondria creatine kinase
mtDNA	Mitochondrial deoxyribonucleic acid
Neh	Nrf2-ECH homology
NF-E2	Nuclear factor erythroid 2
ng	Nanogram
NQO-1	NAD(P)H:quinone oxidoreductase$_1$
Nrf2	NF-E2-related Factor 2

N-terminus	Amino-terminus
PACRG	Parkin co-regulated gene
PAGE	Polyacrylamide gel electrophoresis
PBST	Phosphate Buffered Saline Tween 20
PCR	Polymerase chain reaction
PD	Parkinson's disease
PINK1	PTEN-induced putative kinase 1
PVDF	Polyvinyl difluoride
RING	Really Interesting New Gene
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
siRNA	Small interfering RNA
SNCA	Alpha-synuclein
SNPs	Single-nucleotide polymorphisms
tBHQ	Tert-butylhydroquinone
TEMED	Tetramethylethylenediamine
TOM20	Translocase of outer membrane
TSS	Transcription start site
UBL	Ubiquitin-like domain
µg	Microgram
µM	Micromolar
UGTs	UDP-glucuronosyltransferases
VDAC	Voltage-dependent anion channel

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CHAPTER I

GENERAL INTRODUCTION

1.1 Overview of Parkinson's disease

1.1.1 Discovery of Parkinson's disease

Parkinson's disease (PD) was first documented in Dr. James Parkinson's thesis called "*An Essay on the Shaking Palsy*" in 1817 (Parkinson, 2002). The symptoms and signs of Parkinson's disease were further described and classified by Jean-Martin Charcot in the 18th century (Goetz, 2011). After that, many studies have focused on Parkinson's disease research, which has allowed us to better understand the clinical deficits, pathology, genetic causes, and treatments of Parkinson's disease (Shulman et al., 2011).

1.1.2 Signs and pathology of Parkinson's disease

Clinically, Parkinson's disease is characterized by specific motor changes including "*rest tremor, bradykinesia, rigidity and loss of postural reflexes*" (Jankovic, 2008). Besides typical motor signs, Parkinson's disease patients might also suffer from accompanying non-motor deficits such as "*neuropsychiatric symptoms*", "*sleep disorders*", "*autonomic symptoms*", "*gastrointestinal symptoms*", "*sensory symptoms*", etc. (Chaudhuri et al., 2006).

Pathologically, patients with Parkinson's disease show a progressive degeneration and cell death of dopaminergic neurons in the substantia nigra pars compacta and other groups of neurons (Braak et al., 2003), as well as the presence of Lewy bodies (Halliday and McCann, 2010). The loss of dopaminergic neurons leads to insufficient release of the

neurotransmitter dopamine in the striatum of the forebrain, and results in the motor deficits of Parkinson's disease (Braak et al., 2003; Garrett, 2004).

1.1.3 Treatments of Parkinson's disease

Nowadays, pharmacological treatments such as with "*DA agonists*" (dopamine agonists), "*levodopa (L-DOPA)*", "*Catechol-O-methyltransferase (COMT) inhibitors*", and "*monoamine oxidase (MAO) inhibitors*" are commonly used to relieve the symptoms of Parkinson's disease (Factor, 2008; Kalinderi et al., 2011). The function of dopamine agonists is similar to that of dopamine, in that they can bind to and moduate dopamine receptors, thus resolving the problem of insufficient dopamine in the brain (Factor, 2008; Kalinderi et al., 2011). On the other hand, L-DOPA, which is the precursor of dopamine, can be transformed into dopamine, thus increasing the amount of dopamine available in the striatum (Factor, 2008; Kalinderi et al., 2011). Because L-DOPA can be degraded by the enzyme called Catechol-*O*-methyltransferase (COMT), COMT inhibitors can lengthen the half-life of the L-DOPA by preventing its breakdown (Factor, 2008; Kalinderi et al., 2011). Since dopamine is broken down by monoamine oxidase B (MAO-B), small molecules that inhibit the activity of MAO-B can also increase the amount of the dopamine in the brain (Factor, 2008; Kalinderi et al., 2011).

Besides pharmacological intervention, there are other treatment options for managing motor deficits of Parkinson's disease including surgery (lesions or deep brain stimulation) (Duker and Espay, 2013) and specific physiotherapy/rehabilitation therapies (Horstink et al., 2006).

Importantly, currently available treatments can only relieve symptoms and signs, but they do not prevent or slow down the progression of Parkinson's disease (Factor, 2008; Kalinderi et al., 2011). Moreover, the present treatments have some adverse side effects (Factor, 2008; Kalinderi et al., 2011). Since more and more people are affected with Parkinson's disease due to aging populations around the world (Khandhar and Marks, 2007), there is an urgent need to discover and develop novel, "etiology-based" therapies to stop or delay the development of Parkinson's disease (Singleton et al., 2013).

1.1.4 Genes associated with Parkinson's disease

Currently, we know that there are (at least) two forms of Parkinson's disease called "sporadic Parkinson's disease" of still unresolved etiology (Braak et al., 2003), and "familial (heritable) Parkinson's disease" which is linked to genetic mutations of PD associated genes (Klein and Schlossmacher, 2006; Singleton et al., 2013). Although the majority of diagnosed Parkinson's disease cases are classified as "sporadic" with unknown cause(s) (Braak et al., 2003), an increasing number of studies have found that certain genes are associated with the development of early-onset Parkinson's disease (Klein and Schlossmacher, 2006; Singleton et al., 2013). It has been reported that mutations in the genes encoding alpha-synuclein (*SNCA)* (Polymeropoulos et al., 1997) and leucine-rich repeat kinase 2 (*LRRK2)* (Zimprich et al., 2004) are associated with autosomal dominant Parkinson's disease, while mutations in *Parkin (PARK2*) (Kitada et al., 1998), *DJ-1 (PARK7)* (Bonifati et al., 2003), and *PINK1* (PTEN-induced putative

kinase 1) (Valente et al., 2004) are associated with autosomal recessive Parkinson's disease (Klein and Schlossmacher, 2006; Singleton et al., 2013).

1.2 Introduction of human *Parkin*

1.2.1 Association of human *Parkin* gene (*PARK2*) with Parkinson's disease

Parkin at the PARK2 locus was the first gene identified for autosomal recessive juvenile parkinsonism (AR-JP) (Kitada et al., 1998), which is a prevalent type of heritable Parkinson's disease (Hattori et al., 2000; Klein and Schlossmacher, 2006). In addition to young-onset or early-onset Parkinson's disease, recessive *Parkin* mutations are also found in patients with late-onset Parkinson's disease (Klein et al., 2000; Oliveira et al., 2003). However, it appears that *Parkin* mutations are more common in early-onset cases (Lohmann et al., 2003). According to a previous study, among the cases with juvenile-onset Parkinson's disease (onset age <20 years old), more than 80 percent of these subjects have *Parkin* mutations (Lohmann et al., 2003). On the other hand, among the cases with late-onset Parkinson's disease (onset age >45 years old), only 28 percent of them have *Parkin* mutations (Lohmann et al., 2003).

It has been reported "*more than 100 different parkin mutations*" affecting the *Parkin* gene in Parkinson's patients (Hedrich et al., 2004; Pankratz et al., 2009). Among the various mutations, frequent ones occur in exon 2, 3, 4, and 7 (Hedrich et al., 2004). Mutations in *Parkin* gene linked to the illness result in functional impairment of Parkin protein, which

is thought to contribute to Parkinson's disease in a loss-of-function mechanism (Hedrich et al., 2001; Shimura et al., 2000).

1.2.2 Genomic structure of human *Parkin* gene

The human *Parkin* is located on "*chromosome 6q25.2-27*" (Matsumine et al., 1997), and its genomic size is about 1.4 Mb containing 12 exons (Figure 1) (Asakawa et al., 2001) that encode for a 4.5-Kb (Kilo-base) mRNA (messenger ribonucleic acid) (Kitada et al., 1998). One characteristic of human *Parkin* gene is its large introns (total size is about 1.38 Mb, accounting for approximately 99 percent of the entire human *Parkin* gene), in which the largest is intron 1 that measures 284.1 Kb (Figure 1) (Asakawa et al., 2001; Yu et al., 2005). In 2001, Asakawa *et al*. found that the core promoter is located in a 291-bp (base pair) region of the 5'-flanking sequence of human *Parkin* (Asakawa et al., 2001). While there is "no TATA or CAAT box" located in this region (Asakawa et al., 2001; West et al., 2001), the core promoter region contains several potential transcriptional factor binding sites such as AP4, ATF, GATA1, N-myc, Nrf2 and Sp1 binding sites (Figure 2) (Akiyama 1995; Tsunoda and Takagi, 1999; Asakawa et al., 2001; West et al., 2004; Wingender et al., 1996; Heinemeyer et al., 1998; Heinemeyer et al., 1999).

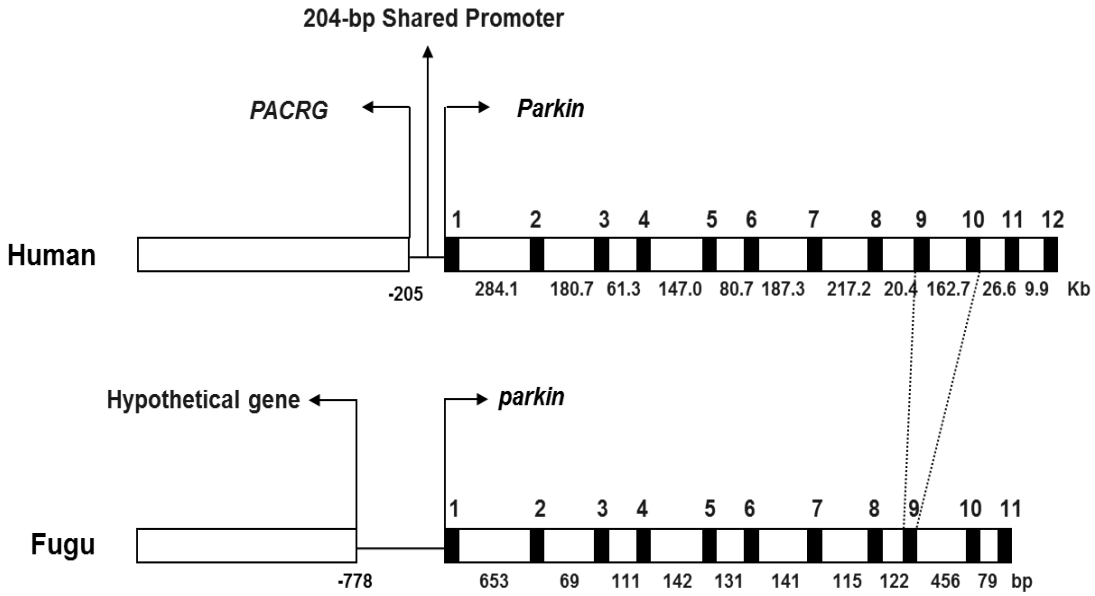


Figure 1. Comparison of the genomic organization of human *Parkin* and Fugu *parkin* genes. Shown is the structure of human *Parkin* and Fugu *parkin* genes (Asakawa et al., 2001; West et al., 2003; Yu et al., 2005). Highlighted are the start codon of the genes (arrows), exons (blocked boxes), introns (open boxes) with their respective sizes indicated below each in Kb or bp. Figure is modified from figure of a previous publication by Yu *et al.* (Yu et al., 2005).

Figure 2. Genomic sequence of the human *Parkin* core promoter region. The numbering is based on the position of the *Parkin* transcription start site (TSS), which is designated as +1. Several potential transcription factor binding sites including two potential Nrf2 sites are located in the promoter region (Akiyama 1995; Tsunoda and Takagi, 1999; Asakawa et al., 2001; West et al., 2004; Wingender et al., 1996; Heinemeyer et al., 1998; Heinemeyer et al., 1999).

Expression of human *Parkin* has been detected in various tissues, predominately in skeletal muscle, heart and brain (Kitada et al., 1998). Moreover, the human *Parkin* gene is expressed in virtually all regions of the brain, including the substantia nigra of the midbrain, thalamus, and hippocampus (Kitada et al., 1998).

In 2003, West *et al*. found a gene called "*Parkin co-regulated gene,* or *PACRG"* located at 204-bp upstream of and antisense to *Parkin* (West et al., 2003). This gene spans over 0.6 Mb and encodes an approximately 1.4 Kb transcript (West et al., 2003). Although *PACRG* and *Parkin* share a "bi-directional promoter" (Figure 1), the "tissue expression profiles" of the two genes are not the same (West et al., 2003). Moreover, *PACRG* might be modulated by the ubiquitin-proteasome pathway (Taylor et al., 2007). *PACRG* might be associated with Parkinson's disease since it is found in Lewy bodies (Taylor et al., 2007).

1.2.3 Functions of human *Parkin*

The human *Parkin* gene encodes the Parkin protein, which is composed of 465 amino acids (Asakawa et al., 2001). Parkin protein contains a "*ubiquitin-like domain*" at its N-terminus (Amino-terminus), an "*IBR (in between RING-finger) motif*" ("*RING: really interesting new gene*"), and two "*RING-finger motifs*" at its C-terminus (carboxyl-terminus) (Kitada et al., 1998; Asakawa et al., 2001; Hristova et al., 2009). Recently, Shaw *et al*. have identified another RING-finger domain and called it RING0 (Hristova et al., 2009). Parkin appears to have multiple protective functions, including: to act as an ubiquitin-E3 ligase for protein degradation to prevent the accumulation of misfolded

proteins (Shimura et al., 2000; Moore, 2006); to maintain the quality of mitochondria (Tanaka et al., 2010; Lim et al., 2011; Winklhofer, 2014); and to prevent cell death by protecting cells from potentially neurotoxic agent (Hyun et al., 2005). These properties are thought to contribute to its protective effect in Parkinson's disease and are presented below.

As one protective function, the Parkin protein is thought to function as an ubiquitin-E3 ligase that targets protein for degradation through the "ubiquitin/proteasome pathway" (Shimura et al., 2000; Haass and Kahle, 2001; Moore, 2006). There are a number of reported putative substrates of Parkin, including the "O-glycosylated α-synuclein", which forms the Lewy bodies detected in Parkinson's disease (Haass and Kahle, 2001; Shimura et al., 2001). It has been reported that Parkin protein loses its ubiquitin ligase activity as a consequence of gene mutations, and such loss of function leads to aggregation of its substrates in neurons (Haass and Kahle, 2001; Shimura et al., 2001).

A second protective function is based on growing evidence that Parkin is involved in the maintenance of normal function and quality of mitochondria (Tanaka et al., 2010; Lim et al., 2011; Winklhofer, 2014). For example, recessive *Parkin* mutations lead to mitochondrial deficits in patients with Parkinson's disease (Muftuoglu et al., 2004) and in animal models (Palacino et al., 2004; Pesah et al., 2004). It has been shown that PD patients have much lower activity of complex I (Muftuoglu et al., 2004). Moreover, a previous study reported that 13 of 14 proteins participitating in "mitochondrial oxidative phosphorylation" or oxidative stress are -unexpectedly- decreased in *parkin* null mice, but only one was elevated (Palacino et al., 2004). Moreover, *parkin* null mice display

impaired respiratory function of mitochondria in the striatum (Palacino et al., 2004). On the other hand, raising *Parkin* expression level increased the "transcription and replication of mitochondrial DNA" in human SH-SY5Y neuroblastoma cells (Kuroda et al., 2006; Rothfuss et al., 2009), a model of dopamine neurons (Xie et al., 2010). Conversely, decreasing *Parkin* expression by small interfering RNA (siRNA) in SH-SY5Y cells had the opposite effect – the "transcription and replication of mitochondrial DNA" was reduced (Kuroda et al., 2006). Using a Chromatin Immunoprecipitation (ChIP) assay, Rothfuss *et al*. demonstrated an interaction between *Parkin* and mtDNA in SH-SY5Y cells, as well as in mouse and human brain tissue (cerebellum) (Rothfuss et al., 2009). Rothfuss *et al*. also found that mtDNA was safeguarded from the harmful effect of oxidative stress in SH-SY5Y cells overexpressing wild-type Parkin (Rothfuss et al., 2009), whereas mtDNA of human fibroblasts were vulnerable to oxidative stress in the absence of wild-type Parkin (Rothfuss et al., 2009). Moreover, *Parkin* interacts genetically with *PINK1* (*PTEN-induced putative kinase 1*) in maintaining the normal function of mitochondria in D. *melanogaster* models; and wild-type Parkin rescued defective mitochondria in cells lacking functional *PINK1* (Park et al., 2006; Exner et al., 2007). Thus, Parkin appears to be important for the normal function and / or homeostasis of mitochondria.

In addition, Parkin over-expression protects SH-SY5Y cells from the toxic effects induced by various substances such as dopamine (Jiang et al., 2004), ceramide (Darios et al., 2003), mutated α-synuclein (A53T) (Petrucelli et al., 2002), "1-methyl-4-phenylpyridinium (MPP^+)" (Hyun et al., 2005), hydrogen peroxide (H_2O_2) (Hyun et al., 2005), and "4-hydroxy-2-trans-nonenal (HNE)" (Hyun et al., 2005). Moreover, Parkin

suppresses the harmful effects caused by over-expression of α-synclein and Pael-R in dopaminergic neurons in *Drosophila* (Yang et al., 2003; Haywood and Staveley, 2006). Thus, Parkin appears to confer resistance to neurotoxic substances / mechanisms that target dopamine neurons in Parkinson's disease.

1.2.4 Alternating human *Parkin* expression level

The multiple protective functions of Parkin suggest that *Parkin* gene expression levels might be one of the factors influencing the survival rate of cells in the brain stem (Ulusoy and Kirik, 2008). Therefore increasing the level of wild-type Parkin might be able to rescue cells at risk, which in turn would stop or slow the development of Parkinson's disease (Ulusoy and Kirik, 2008). To increase *Parkin* expression, it is important to identify the cis- and trans-acting transcriptional regulatory elements and signaling pathways involved in its expression: cis-acting elements are the binding sequences for trans-acting factors (transcription factors); in turn, transcription factors can act as activators or inhibitors of gene expression. In this thesis, I will characterize potential cis-acting elements and the transcription factors that recognize them in the regulation of the *Parkin* gene.

1.2.5 Transcriptional regulation of human *Parkin* gene

Several modifiers of *Parkin* gene transcription have been reported. Dopamine and "1-methyl-4-phenylpyridinium (MPP+)" increase the expression of Parkin in SH-SY5Y cells

through activating the transcription of *Parkin* (Yang et al., 2006). In addition, "ATF4 (Activating Transcription Factor 4)" induced the transcription of *Parkin* by binding to the ATF4 binding sequence located in the promoter region of *Parkin* (Bouman et al., 2011; Sun et al., 2013). On the other hand, N-myc transcription factor inhibits the transcription of *Parkin* through interacting with the N-myc binding sequence located at the promoter region of *Parkin* (West et al., 2004). Thus, there are several potential mechanisms regulating *Parkin* expression.

In addition, genetic changes have been identified in the *Parkin* gene that alter its expression. West *et al*. found nine single-nucleotide polymorphisms (SNPs) in the human *Parkin* gene promoter (West et al., 2002). Among these SNPs, "-258 T/G and -277 A/G" are located in the *Parkin* promoter, and -258 T/G polymorphism is associated with idiopathic Parkinson's disease (West et al., 2002). Moreover, -258 T/G polymorphism reduces *Parkin* promoter activity, which in turn affected *Parkin* gene expression, suggesting that *Parkin* gene expression might contribute to the risk of idiopathic PD (West et al., 2002). Tan *et al* also found that the transcriptional activity of the -258T, but not the -258G variant, is significantly increased in SH-SY5Y cells exposed to hydrogen peroxide or MG132 (proteasome inhibitor) (Tan et al., 2005). Moreover, their case control study involving 386 Parkinson's disease patients and 367 controls showed that -258 T/G polymorphism was associated with the development of Parkinson's disease (Tan et al., 2005). Thus, altered transcriptional regulation of *Parkin* may predispose humans to Parkinson's disease.

1.2.6 Challenges in studying the transcriptional regulation of human *Parkin*

As of today, limited information is available regarding the mechanisms regulating *Parkin* gene transcription (Yu et al., 2005). Examining the potential transcriptional regulatory elements and pathways of human *Parkin* has been difficult because of its large size (Asakawa et al., 2001; Yu et al., 2005). Human *Parkin* gene contains large introns, making it the second largest known human gene; and 14 bacterial artificial chromosome (BAC) clones were required to cover and identify the entire *Parkin* gene (Kitada et al., 1998; Asakawa et al., 2001). Fortunately, Yu *et al* described an ortholog of human *Parkin* in Fugu fish (Fugu *parkin* gene) (Yu et al., 2005). While the "genomic structure", gene function and "tissue expression pattern" of Fugu *parkin* is similar to that of human *Parkin*, the Fugu *parkin* gene has a much smaller size than the human *Parkin* gene (Yu et al., 2005). Therefore, the Fugu *parkin* gene might be a suitable model containing the essential cis-acting DNA (deoxyribonucleic acid) elements for studying the transcription factors and signaling pathways involved in *parkin* expression (Yu et al., 2005).

1.3 Comparison of Fugu *parkin* to human *Parkin*

The Fugu *parkin* gene, which contains 11 exons and 10 introns, is similar to human *Parkin* in "genomic structure*"* and in the total length of its exons (Fugu *parkin* 1.446 kb, human *parkin* 1.395 kb) (Figure 1) (Yu et al., 2005). However, Fugu *parkin* (4 kb) is about 350 times smaller in size than human *Parkin* (1.4 Mb) because the introns of Fugu *parkin* are small (Figure 1) (Yu et al., 2005). According to the "*TRANSFAC*" transcription factor database, "*TFBIND*" and "*TFSEARCH*" softwares, the entire 5'-

flanking region of Fugu *parkin* contains several potential transcription factor binding sites, such as for AP4, MZF1, SOX5, SRY, and Nrf2 (Figure 3) (Wingender et al., 1996; Heinemeyer et al., 1998; Heinemeyer et al., 1999; Tsunoda and Takagi, 1999; Akiyama, 1995; Yu et al., 2005).

Moreover, similar to human *Parkin*, Fugu *parkin* is also expressed in a variety of tissues including the brain (Yu et al., 2005). In addition, the protein sequences of Fugu parkin and human Parkin are quite similar (the amino acid identity is 53% for the entire sequence; and 61% at the RING finger motifs) (Yu et al., 2005). Furthermore, most of the regions that are critical for protein function are conserved between Fugu and human Parkin proteins and both proteins have ubiquitin ligase function (Yu et al., 2005). Based on the similarities in "genomic structure", "tissue expression pattern", and protein function between human *Parkin* and Fugu *parkin*, the authors suggested that expression of the two genes might be controlled by a similar mechanism (Yu et al., 2005). The smaller size of the Fugu *parkin* gene compared to the human orthologue makes it more amenable for characterization of promoter function; hence I have focused on characterization of the Fugu *parkin* to shed light on *Parkin* regulation.

Yu *et al*. also identified *pacrg* gene in Fugu fish, which is considered an ortholog of the human *PACRG* gene (Yu et al., 2005). However, Fugu *pacrg* is not located antidromal and upstream of Fugu *parkin* as seen in the human gene, and as a result, the two Fugu genes do not share a bi-directional promoter (Yu et al., 2005)

-779 CATCAGTTTT TTCATTATAC TTAGTTGTCT TCATGCTAAG ACTAAATTTG

SRY

-729 CGATTGTTGT AAGTGTAAAT TCTTGGAATC CAACGTATAA CTACCGGTAG

SOX5 SRY

-679 TTGGAAAGAA ATGGAATTGG AATAACTGGT TTATTTTAAT CCCCTCATAA

SRY MZF1

-629 ATATAGAAAT GAGCAAATCA AGATTGGCTT GATATGTGGC GGACCAAGCA

-579 GGAAATCGAC TGATCAATTG TAGTGCCCTG ACTGTTTCAT GTAAAATTTG

-529 CAACGAGTAT GTCCTAATTA AAAGCCACTT TTAATCATCC TATATTTAAT

-479 GATTAACAGT TTAGTGGGAC GTCAGCTTGT TGACATTAGA AGTGCATGTT

-429 AAGCGTTTCA TTTAGTGCTA GTTCTGATCA TTGACGTTAA TCAATAACAC

-379 CGTGATGGGA TCCGCGATTG GCTGTGGATC ATTAAGGTGT GTATCTGCTG

-329 GACGTCTCAC CACCGGCGGC CCAACGCTGC CGCTTGTTGT CCGCTGCAAG

-279 CCGCTGCTGT C CGGACTGTC CGCCATCTGT AGCCTCGAAA AGATACCGAT

AP4

-229 AGCCGAGAAA AACGGCGTCC GTCAGGTTTC GCTCGGCTGT CCCTCACAAA

-179 AACACACAAA GACGGGGAGA AAAAAGAAAA CAAATTTATT AAAATTCATA

MZF1 SRY

-129 GGGGTATCCA CTTCCGGTGC TGAAACTACG TAATGTCTAC GCGACTAAAT

Nrf2

-79 TGTAGTTTTC TTGAAAACGT AGTTGTTTCG TTTGTAGTCT GATTGTGACT

parkin TSS

-29 ACAGTACCCA TTATGCATCG GTACAGGCC**G** **ACCGTAAACA CGTTTGGAGC**

+1

+22 **CACGAAGGTC GACGGGGCTG ATGATCG**GTG

+42

Figure 3. Genomic sequence of the 5'-flanking region of Fugu *parkin*. The numbering is based on the position of *parkin* transcription start site (TSS), which is designated as +1. Several potential transcription factor binding sites such as AP4, MZF1, Nrf2, SOX5, and SRY are located in that region (Wingender et al., 1996; Heinemeyer et al., 1998; Heinemeyer et al., 1999; Tsunoda and Takagi, 1999; Akiyama, 1995; Yu et al., 2005).

1.4 Nrf2 transcription factor

In order to identify potential regulatory DNA elements in the *Parkin* gene, I conducted a database search of the Fugu and human *Parkin* promoter sequences. This search yield strong consensus elements for the transcription factor Nrf2 as described below (Wingender et al., 1996; Heinemeyer et al., 1998; Heinemeyer et al., 1999; Tsunoda and Takagi, 1999; Akiyama, 1995) and identified using the "*TFSEARCH*" (Akiyama, 1995) and the "*TFBIND*" softwares (Tsunoda and Takagi, 1999). Nrf2 consensus sequences were not only found in the promoter sequences of human *Parkin* and Fugu *parkin*, but also detected in the promoters of mouse *parkin* and rat *parkin* (Wingender et al., 1996; Heinemeyer et al., 1998; Heinemeyer et al., 1999; Tsunoda and Takagi, 1999; Akiyama, 1995). Because Nrf2 consensus sequences are conserved in mammalian *parkin* genes, it is possible that the Nrf2 transcription factor might be involved in the regulation of *parkin* gene expression. Therefore, in this study, I focused on testing whether the potential Nrf2 binding sites located in the Fugu *parkin* and human *Parkin* promoter sequences were involved in activation of the *parkin* gene.

1.4.1 Consensus Sequence of Nrf2 transcription factor

A strong consensus DNA element for Nrf2 was identified in the Fugu and human *Parkin* promoter regions (Asakawa et al., 2001; Yu et al., 2005; Wingender et al., 1996; Heinemeyer et al., 1998; Heinemeyer et al., 1999) using the "*TFSEARCH*" (Akiyama, 1995) and the "*TFBIND*" softwares (Tsunoda and Takagi, 1999). According to the "*TRANSFAC*" transcription factor database and "*TFBIND*" software, the consensus

sequence of Nrf2 is "*ACCGGAAGNS*" in which N represents A, T, C or G; and S represents C or G (Figure 4) (Wingender et al., 1996; Heinemeyer et al., 1998; Heinemeyer et al., 1999; Tsunoda and Takagi, 1999). Two putative Nrf2 binding sites were detected in the promoter region of human *Parkin* while one Nrf2 binding site was detected in the putative promoter region of Fugu *parkin* (Figure 4) (Asakawa et al., 2001; Yu et al., 2005; Wingender et al., 1996; Heinemeyer et al., 1998; Heinemeyer et al., 1999; Tsunoda and Takagi, 1999; Akiyama, 1995). Thus, Nrf2 could be a potential regulator of *Parkin* gene expression.

Consensus Nrf2 sequence: ACCGGAAGNC/G (N = any base pair)

a. Putative Nrf2 sites in human *Parkin* promoter

Nrf2(1): -227 to -217

```
Forward strand   5′… GCCTCCCGGT …3′
Reverse strand   5′… ACCGGGAGGC …3′
                     ::::: ::::
Consensus Nrf2       ACCGGAAGNC
```

Nrf2(2): -278 to -268

```
Forward strand   5′… GCGGGAAGAG …3′
                      : :::::::
Consensus Nrf2       ACCGGAAGNG
```

b. Putative nrf2 site in Fugu *parkin* promoter

nrf2: -161 to -151

```
Forward strand   5′… CACTTCCGGT …3′
Reverse strand   5′… ACCGGAAGTG …3′
                     ::::::::::
Consensus Nrf2       ACCGGAAGNG
```

Figure 4. Potential Nrf2 binding sites in the *Parkin* promoter. Consensus sequence of Nrf2 is "*ACCGGAAGNC/G*"; two potential Nrf2 binding sites are located in the human *Parkin* promoter, while one potential Nrf2 binding site is located in the 5'-flanking region of Fugu *parkin* (Asakawa et al., 2001; Yu et al., 2005; Wingender et al., 1996; Heinemeyer et al., 1998; Heinemeyer et al., 1999) identified using the "*TFSEARCH*" (Akiyama, 1995) and the "*TFBIND*" softwares (Tsunoda and Takagi, 1999). The sequences comparisons were carried out using the sequence alignment program "*ALIGN*" ("*GENESTREAM SEARCH network server IGH Montpellier, France"*) available at "*http://xylian.igh.cnrs.fr/bin/align-guess.cgi*" (Pearson et al., 1997)

1.4.2 Structure and function of Nrf2 transcription factor

The transcription factor "*Nrf2 (NF-E2-related Factor 2)*" is one of the "*basic leucine zipper (bZIP) transcription factors*" belonging to the "*Cap'n'Collar (CNC) families*" (Moi et al., 1994; Motohashi et al., 2002). The Nrf2 protein, which is composed of 605 amino acids, contains six domains named "*Neh1 to Neh 6 (Nrf2-ECH homology)*" (Itoh et al., 1999; Baird and Dinkova-Kostova, 2011). The Neh1 domain is the bZIP domain that is required for Nrf2 to heterodimerize with small Maf proteins (Marini et al., 1997; Itoh et al., 1999; Baird and Dinkova-Kostova, 2011); the Neh2 domain binds to "*Keap1 (Kelch-like ECH-associated protein1)*" (Itoh et al., 1999); the Neh3 domain interacts with a transcriptional co-activator called the "*CHD6 (a chromo-ATPase/helicase DNA binding protein*" (Nioi et al., 2005); Neh4 and Neh5 domains interact with "*CBP (CREB (cAMP Responsive Element Binding protein) Binding Protein)*" (Katoh et al., 2001), while Neh6 is implicated in regulating the degradation of Nrf2 without the involvement of Keap1 (McMahon et al., 2004).

The Nrf2 transcription factor is expressed abundantly in organs responsible for detoxification such as kidney and lung (Moi et al., 1994). Nrf2 plays an important role in defending cells from xenobiotics by increasing the expression of genes encoding for cytoprotective proteins such as "*NAD(P)H:quinone oxidoreductase$_1$ (NQO$_1$)*" (Venugopal and Jaiswal, 1996), "*heme oxygenase-1 (HO-1)*" (Alam et al., 1999), "*glutathione S-transferase (GST*)" (Hayes et al., 2000), "*UDP-glucuronosyltransferases (UGTs)*" (Yueh and Tukey, 2007), "*Multidrug resistance-associated proteins (Mrps)*" (Maher et al., 2007), among many others. Intriguingly, about 650 "inducible target

genes" of the Nrf2 transcription factor have been detected (Malhotra et al., 2010). In response to the stressors, Nrf2 pathway is activated and Nrf2 translocates from cytoplasm to nucleus and bind to the *"antioxidant responsive element*" ("*ARE consensus sequence*": "*TGACNNNGC*") of genes encoding cell protective proteins to increase their expression (Motohashi et al., 2002). Besides, Nrf2 can also bind to the *NF-E2 ("nuclear factor erythroid 2")* binding sequence "*(T/C)GCTGA(C/G)TCA(T/C)"* (Andrews et al., 1993; Moi et al., 1994). Nrf2 does not bind to ARE sequence alone, but forms a heterodimer with small Maf proteins when binding to the ARE recognition element (Marini et al., 1997; Motohashi et al., 2002). Besides small Maf proteins, other proteins such as ATF-4 can heterodimerize with Nrf2 and bind to ARE sequence (He et al., 2001; Motohashi et al., 2002). The Nrf2 transcription factor is considered a strong transcriptional inducer of its target genes (Katoh et al., 2001) and when compared to other CNC family members including NF-E2 (Andrews et al., 1993), Nrf1 (Chan et al., 1993), and Nrf3 (Kobayashi et al., 1999), Nrf2 is found to be the most effective activator (Katoh et al., 2001). Thus, Nrf2 is a key mediator of cell protection (Baird and Dinkova-Kostova, 2011).

1.4.3 Regulation of Nrf2 transcription factor

It is now clear that there are 10 classes of chemicals that can induce Nrf2 transcription factor: "*(1) oxidisable diphenols, phenylenediamines and quinones, (2) Michael reaction acceptors (olefins or acetylenes conjugated with electron-withdrawing groups), (3) isothiocyanates and sulfoxythiocarbamates, (4) thiocarbamates, (5) dithiolethiones, (6) conjugated polyenes, (7) hydroperoxides, (8) trivalent arsenicals, (9) heavy metals and*

(10) vicinal dimercaptans" (Baird and Dinkova-Kostova, 2011). Widely used Nrf2 inducers including the "tert-butylhydroquinone (tBHQ)*"* belong to the class 1 (Baird and Dinkova-Kostova, 2011). In addition to tBHQ, "diethyl maleate (DEM)" and "L-buthionine-(S,R)-sulfoximine (BSO)" are typical activators of Nrf2 pathway (Lee et al., 2001; Katsuoka et al., 2005; Lee et al., 2008). Moreover, copper II chloride ($CuCl_2$) and other transition metals can further enhance the induction effect of the phenolic compounds (e.g. tBHQ) by oxidizing the phenolic compounds to quinones; these quinones then oxidize the cysteine residues in Keap1, which are important for Keap1 interaction with and subsequent degradation of Nrf2 (Dinkova-Kostova and Wang, 2010; Wang et al., 2010). However, the underlying mechanism of how these chemicals activate Nrf2 transcription factor is still incompletely understood (Baird and Dinkova-Kostova, 2011). Several models of Nrf2 induction have been proposed based on experimental results of different studies; and such models have been described in detail previously (Baird and Dinkova-Kostova, 2011).

The model which is supported by many experimental results is the "*Dissociation of Keap1 and Cullin 3 model*" (Baird and Dinkova-Kostova, 2011). Keap1 is composed of 624 amino acids which comprises 3 domains including the "*BTB dimerisation domain*" (BTB: "*Broad-Complex, Tramtrack, and Bric à brac*") which mediates Keap1 interaction with the "*Cullin 3 (Cul3)-based ubiquitin ligase*"; the "*cysteine-rich IVR domain (Intervening region)*" harboring the cysteine residues, and the "*Kelch domain*" where the interaction between Keap1 with Nrf2 takes place (Baird and Dinkova-Kostova, 2011). In the "*Dissociation of Keap1 and Cullin 3 model*", Keap1 acts as a repressor of Nrf2: Under the non-induced condition, Keap1 functions as a "*substrate adaptor protein*" by

binding to Cul3 through the "*BTB dimerisation domain*" and to the Nrf2 through the "*Kelch domain*" (Baird and Dinkova-Kostova, 2011). In this way, Nrf2 is ubiquitinated and degraded via the ubiquitin–proteasome pathway (Baird and Dinkova-Kostova, 2011). Therefore, Nrf2 is rapidly removed and only a low level of Nrf2 is expressed in cytoplasm under non-induced conditions (Baird and Dinkova-Kostova, 2011). In particular, it has been reported that the half-life of Nrf2 is less than 15 minutes (Stewart et al., 2003). On the other hand, under the induced condition, the inducers break apart the association between Keap1 and Nrf2 by modifying the cysteine residues in Keap1 that are important for the binding between Keap1 and Nrf2 (Baird and Dinkova-Kostova, 2011). After dissociation from Keap1, Nrf2 transcription factor translocates to the nucleus and binds to the ARE consensus sequence of its target gene to up-regulate gene expression in order to protect the cells from xenobiotics (Sykiotis and Bohmann, 2010; Baird and Dinkova-Kostova, 2011).

Besides Keap1, the expression level of small Maf protein might be crucial for regulating the Keap1-Nrf2 pathway (Igarashi et al., 1994; Motohashi et al., 2002). Besides functioning as a transcriptional activator by dimerizing with Nrf2 to increase the expression of Nrf2 target genes, the small Maf protein might also act as a transcriptional repressor by competing with Nrf2, forming a homodimer with another small Maf (Igarashi et al., 1994; Nagai et al., 1998; Motohashi et al., 2002). Since homodimers of small Maf inhibit transcription, the expression level of small Maf protein is an important factor for regulating the expression of Nrf2-targeted genes (Igarashi et al., 1994; Nagai et al., 1998; Motohashi et al., 2002).

1.4.4 Potential function of Nrf2 in Parkinson's disease

An increasing number of studies have suggested that pro-oxidant forces generated by "reactive oxygen species" (ROS) might be associated with neuronal death in Parkinson's disease (Jenner, 2003). ROS are produced in the brain by various sources: for instance, impaired complex I (Pitkanen and Robinson, 1996) and dopamine metabolism (Chen et al., 2008) generate ROS in the brain (Cuadrado et al., 2009). Markers of oxidative damage, such as increased levels of carbonyl modified protein (Floor and Wetzel, 1998), "lipid hydroperoxides" (Dexter et al., 1994), "8-hydroxyguanine" (Boiteux and Radicella, 1999), and proteins modified by the "4-hydroxy-2-nonenal" (Yoritaka et al., 1996) have been detected in the substantia nigra of Parkinson's brain tissue; suggesting that oxidative damage occurs in Parkinson's disease, and this oxidative damage might impair the normal function of dopamine neurons (Jenner, 2003).

More and more studies suggest that Nrf2 might confer neuronal protection, and that activation of Nrf2 might serve as a potential therapy for preventing the development and progression of Parkinson's disease (Cuadrado et al., 2009). First of all, besides being expressed in organs responsible for detoxification (Moi et al., 1994), Nrf2 is also expressed in brain cells including neurons, astrocytes and other glial cells (Lee et al., 2003; Shih et al., 2003; Kraft et al., 2004; Cuadrado et al., 2009). Moreover, in response to oxidative stress, Nrf2 transcription factor translocates to the nucleus to bind to the ARE sequence of antioxidant genes to up-regulate their expression (Baird and Dinkova-Kostova, 2011). Indeed, a previous study of the cellular localization of Nrf2 in human brain tissues found that Nrf2 tends to localize in the nucleus of substantia nigra neurons

of Parkinson's disease brain tissues (Ramsey et al., 2007). Furthermore, Nrf2 offered protection to cortical neurons against hydrogen peroxide and glutamate (Kraft et al., 2004); and protected mice from neurotoxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) (Burton et al., 2006). Moreover, *DJ-1*, which is a gene associated with Parkinson's disease, was able to prevent the degradation of Nrf2 (Clements et al., 2006; Cuadrado et al., 2009). Since many of the target genes of Nrf2 have cell protective functions (Cuadrado et al., 2009) and because Parkin also has cytoprotective effects (Ulusoy and Kirik, 2008), Nrf2-mediated regulation of Parkin could be a protective mechanism in Parkinson's disease (Cuadrado et al., 2009). Because the Nrf2 consensus sequence is found in the promoter regions of mammalian *parkin* genes including human *Parkin* and mouse *parkin*, as well as in Fugu *parkin* (Akiyama 1995; Tsunoda and Takagi, 1999; Wingender et al., 1996; Heinemeyer et al., 1998; Heinemeyer et al., 1999), it will be important to test whether the expression of *Parkin* is regulated by the Nrf2 transcription factor and its activation pathway.

1.5 Hypothesis

In this study, I hypothesize that Fugu fish and human *Parkin* gene promoters share cis- and trans-acting transcriptional regulatory mechanisms that control the expression of *Parkin*; and that identification of these elements in the Fugu *parkin* gene will allow for the up-regulation of mammalian *Parkin* gene in cell culture conditions. One of the hypothesized upregulators of mammalian *Parkin* is Nrf2. In order to test this hypothesis,

I have examined whether Nrf2 regulates human or Fugu *parkin* transcription using luciferase reporter constructs and co-transfection with Nrf2.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

Constructs: Fugu *parkin* genomic DNA, Flag-tagged human parkin and flag-tagged Fugu parkin constructs were provided by Dr. Kah-Leong Lim's laboratory (National Neuroscience Institute, Singapore) (Yu et al., 2005). Fugu parkin-EGFP-pDrive construct was generated by Sandra Noble and Shannan May-McNally (May-McNally et al., 2010) in Dr. Marc Ekker's laboratory (Department of Biology, University of Ottawa). An eGFP-encoding cDNA plasmid was available in Dr. Michael Schlossmacher's laboratory. pcDNA3.1 (-) vector was from Invitrogen Corporation. pCMV6-XL5 vector was from Origene. pGL4.20 [*luc2*/Puro] plasmid and pRL CMV Renilla Luciferase construct were from Promega. "*pcDNA-Myc-Nrf2 plasmid*" ("*Addgene Plasmid 21555*") and "*pcDNA3-HA2-Keap1*" ("*Addgene Plasmid 21556*") were generated by Furukawa *et al.* (Furukawa and Xiong, 2005) and were purchased from Addgene. Primers for PCR, site-directed mutagenesis and DNA sequencing were from Operon and Sigma.

Enzymes: Restriction enzymes including AgeI, BglII, BsmBI, BspEI, EcoRI, HindIII, MluI, NruI, SacI, SalI, SnaBI and T4 DNA ligase were from New England Biolabs. Klenow enzyme was from Roche.

Cells: Cell lines including CHO cells (CRL-2243) and HEK-293 cell (CRL-1573) were purchased from ATCC. Subcloning Efficiency™ DH5α™ Chemically Competent E. coli and ElectroMAX™ DH5α-E™ Competent E. coli Cells were purchased from Invitrogen. Mouse primary embryonic neurons were isolated from mice (C57BL/6) provided by Dr. David Park's laboratory (Department of Cellular and Molecular Medicine, University of Ottawa).

Chemicals: L-buthionine-sulfoximine (BSO) (purity ≥ 97%), diethyl maleate (DEM) (purity 97 %), *tert*-Butylhydroquinone (tBHQ) (purity 97%), copper (II) chloride ($CuCl_2$) (purity 97%), and dimethyl sulfoxide (DMSO) (purity ≥ 99.9%) were from Sigma-Aldrich.

Antibodies: Anti-Parkin (PARK8) monoclonal antibody (#MAB5512) was from Millipore, and anti-Parkin monoclonal antibody (P6248) was from Sigma. Anti-Flag antibody was from Sigma. Anti-GFP monoclonal antibody (#632381) was from Clontech. Anti-Myc-Tag (9B11) monoclonal antibody (#2276) was from Cell Signaling. Anti-Keap1 polyclonal antibody (#ab31973), anti-Heme Oxygenase 1 (HO-1) monoclonal antibody (#ab13248), and anti-beta-actin monoclonal antibody (ab8226) were from Abcam.

Reagents: Lipofectamine 2000 was from Invitrogen. Paraformaldehyde was from Electron Microscopy Sciences. ProLong® Gold antifade reagent was from Invitrogen. Dual-Luciferase Reporter Assay System was from Promega Corporation. PureYield Plasmid Maxiprep System was from Promega Corporation. Thermo Scientific Pierce BCA Protein assay kit was from Fisher Scientific. NucleoSpin Extract II kit and NucleoSpin Plasmid QuickPure kit were from MACHEREY-NAGEL. NuPAGE Bis-Tris Pre-Cast gels were from Invitrogen.

MPB-Parkin: Constructs of MBP-Parkin cDNAs (full length Parkin, IBR-RING2, 311-stop, 416-stop) in the PMAL-p2T were a gift from Dr. Keiji Tanaka (Matsuda et al., 2006). E.coli strain BL21(DE3) codon-plus (RIL) strain was from Stratagene. Gravity-

flow chromatography column was from Bio-Rad. Amylose Magnetic Beads was from New England BioLabs. Silver Staining Kit (Protein) was from Sigma-Aldrich.

Antibodies for mitochondria study: Aconitase (ACO2) rabbit polyclonal antibody (#11134-1-AP) was from ProteinTech Group Inc.; Creatine kinase MT (MtCK) mouse monoclonal antibody (#ab76506) was from Abcam; Translocase of outer membrane (Tom20) mouse monoclonal antibody (#612278) was from BD Transduction Laboratories; Voltage-dependent anion channel (VDAC) mouse monoclonal antibody (#MSA03) was from MitoSciences; Calnexin antibody mouse monoclonal antibody (#MAB3126) was from Millipore; Glucose-regulated protein 78 (GRP78) mouse monoclonal antibody (#610978) was from BD Transduction Laboratories^{TM}; Cathepsin D rabbit polyclonal antibody (#2284) was from Cell Signaling. β-glucosidase (GBA) rabbit polyclonal antibody (#sc-32883) was from Santa Cruz.

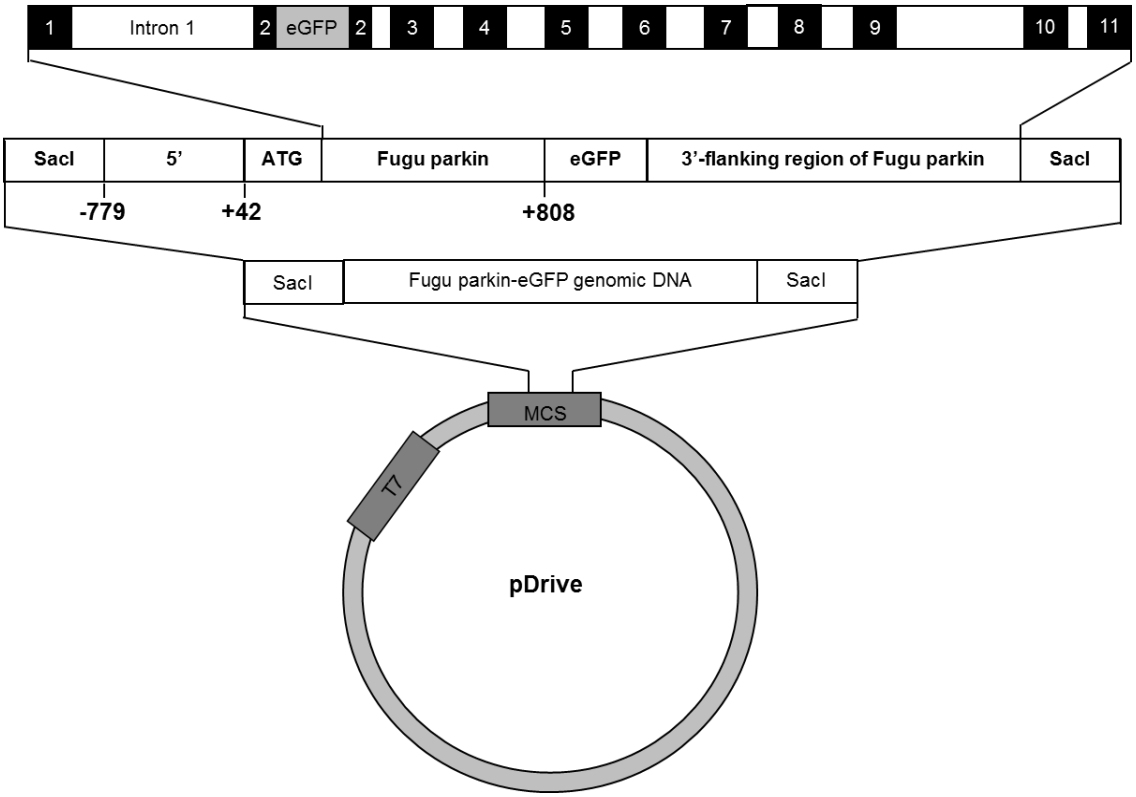
2.2 Methods

2.2.1 Cloning of constructs

2.2.1a Cloning of Fugu parkin-eGFP constructs

Since I wanted to investigate the cis- and trans- transcriptional factors of *Parkin* gene, I used the Fugu *parkin* genomic clone (Yu et al., 2005) and the Fugu parkin-eGFP-pDrive construct (May-McNally et al., 2010). The procedures for cloning the Fugu parkin-eGFP-pDrive construct were described in detail in Shannan May-McNally's bachelor thesis

(May-McNally et al, 2010). Briefly, the entire Fugu *parkin* genomic sequence containing the 5'-flanking region, 11 exons, 10 introns and the 3'-flanking region of Fugu *parkin* was amplified from the Fugu *parkin* clone (Yu et al., 2005) by PCR (polymerase chain reaction) using primers flanking with *Sac*I registration sites; then PCR product of Fugu *parkin* genomic sequence was subcloned into pDrive vector by TA cloning; then eGFP (enhanced Green Fluorescent Protein) complementary DNA (cDNA) sequence was inserted into exon 2 of Fugu *parkin*'s genomic sequence using *Bgl*II restriction enzyme digestion, and ligated (May-McNally et al., 2010) (Figure 5).



Fugu parkin (**MIVFVRYNLGPEVVVELQEEATVAELKEVVGQQQGVQPD**)-eGFP

Amino acids 1-39 of Fugu parkin

Figure 5. Schema of Fugu parkin-eGFP genomic DNA clone. The entire genomic sequence of Fugu *parkin* contains 11 exons, 10 introns, and the 5'-flanking region as well as the 3'-flanking region of Fugu *parkin* (Yu et al., 2005). EGFP-encoding reporter cDNA was subcloned into exon 2 of the genomic Fugu *parkin* sequence (May-McNally et al., 2010; Yu et al., 2005). The Fugu parkin-eGFP fusion protein consists of amino acids 1-39 of Fugu parkin tagged with eGFP (Yu et al., 2005). Figure modified from Shannan May-McNally's bachelor thesis (May-McNally et al, 2010); and with reference to previously publication (Yu et al., 2005) and the pDrive vector map provided by the manufacturer available at *http://www.qiagen.com/images/catalog/1785.gif*.

I first needed to determine whether the promoter region of Fugu *parkin* could drive its expression under cellular conditions. Therefore, based on the Fugu parkin-eGFP-pDrive construct (May-McNally et al, 2010), I further cloned several Fugu parkin-eGFP constructs with or without CMV (Cytomegalovirus) promoter, and with or without the Fugu *parkin* 5'-flanking region for mammalian cell expression. For simplicity, I refer to these constructs by the promoter and eGFP, although the eGFP is fused to the initial coding sequence of Fugu *parkin* in all constructs. CMV-Fugu parkin-eGFP-pcDNA3.1(-) construct (Figure 6A): the whole Fugu parkin-eGFP sequence, which was obtained by digesting the Fugu parkin-eGFP-pDrive with *Eco*RI (New England Biolabs), was subcloned into pcDNA3.1(-) mammalian expression vector containing CMV promoter (Invitrogen) at *Eco*RI site and ligated using T4 ligase (New England Biolabs). Fugu parkin-eGFP-pcDNA3.1(-) construct (Figure 6B): the whole Fugu parkin-eGFP sequence, which was obtained by digesting the Fugu parkin-eGFP-pDrive with *Mlu*I and *Hind*III (New England Biolabs), was subcloned into pcDNA3.1(-) vector without CMV promoter at *Mlu*I and *Hind*III sites and ligated by T4 ligase. CMV-eGFP-pCMV6-XL5 (Figure 6C): the Fugu parkin-eGFP coding sequence lacking the 5'-flanking region of Fugu *parkin*, which was obtained by digesting the Fugu parkin-eGFP-pDrive with *Sal*I (New England Biolabs), was subcloned into pCMV6-XL5 vector containing CMV promoter (Origene) at *Sal*I site and ligated by T4 ligase. Each ligated plasmid was then transformed into *E.coli* bacterial cells (Electrocompetent or chemically competent) (Invitrogen) for replication. Then, replicated plasmid was harvested and purified from *E.coli* bacterial cells by using NucleoSpin Plasmid QuickPure kit (MACHEREY-NAGEL) according to the manufacturer's instruction. In order to select the correct (positive) clone of Fugu

parkin-eGFP construct, the purified plasmid sequence was checked by restriction analysis and by DNA sequencing. DNA sequencing reactions were processed and carried out by the *StemCore Laboratories* at the *Ottawa Hospital Research Institute.* After confirming the correct clone, sufficient amounts of purified Fugu parkin-eGFP construct for cell transfection were prepared by using PureYield™ Plasmid Maxiprep System (Promega) according to manufacturer's instruction.

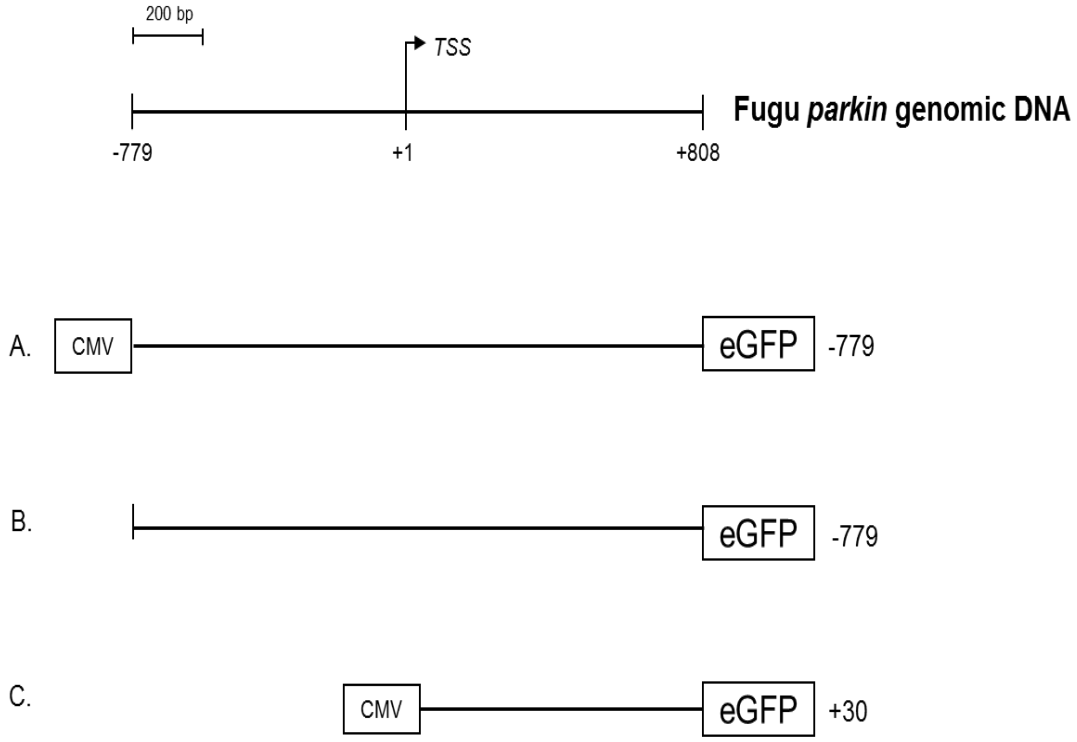


Figure 6. Schema of Fugu parkin-eGFP constructs. (A) CMV-Fugu parkin-eGFP-pcDNA3.1(-) containing the entire 5'-flanking region of Fugu *parkin* with CMV promoter; (B) Fugu parkin-eGFP-pcDNA3.1(-) containing the entire 5'-flanking region of Fugu *parkin* without CMV promoter; (C) CMV-eGFP-pCMV6-XL5 lacking the 5'-flanking region of Fugu *parkin* with CMV promoter. Note that in all constructs eGFP is fused downstream of the initial Fugu *parkin* coding sequence. Promoter map with scale bar is presented schematically with reference to a previous publication (Ou et al., 2000).

2.2.1b Cloning of Fugu parkin-Luciferase constructs

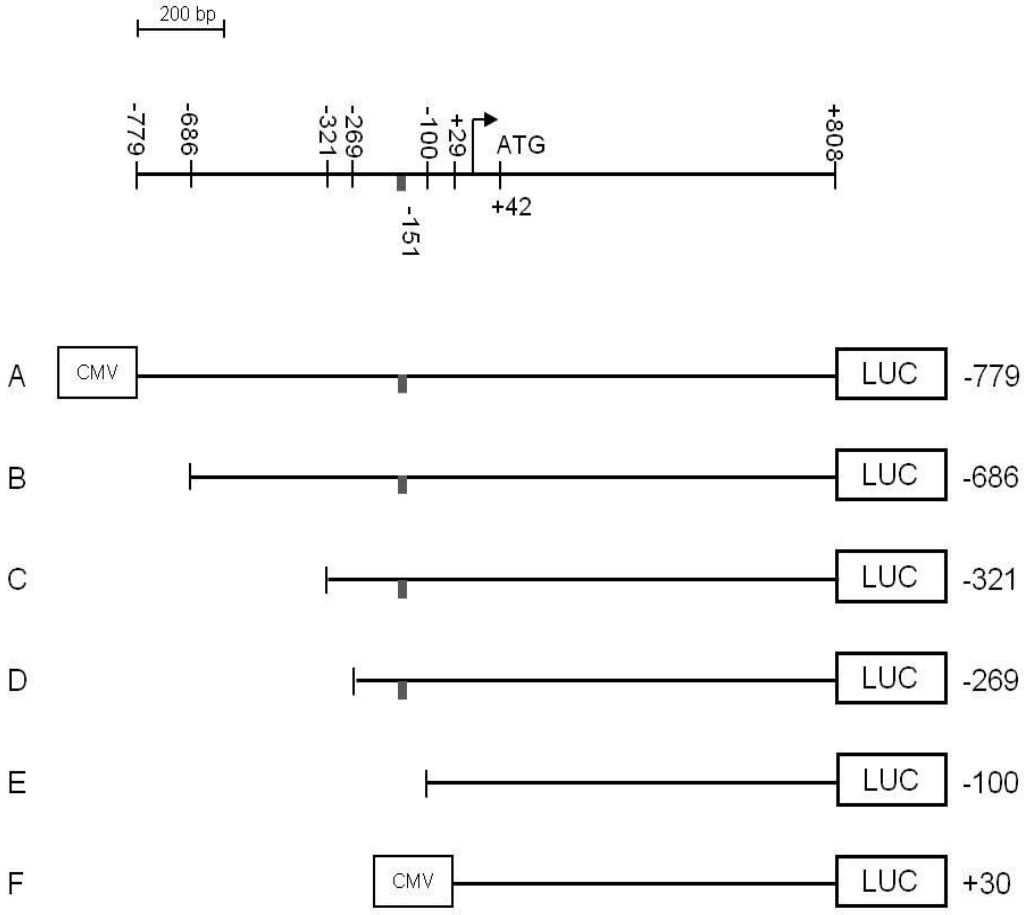
In order to further determine the promoter region of Fugu *parkin,* I cloned several Fugu parkin-Luciferase constructs containing various deletions of the 5'-flanking region of Fugu *parkin* to test their promoter activities. For simplicity, I refer to these constructs by the promoter name and luciferase, although the luciferase is fused to the initial sequence of Fugu *parkin* in all constructs.

First, I cloned the Fugu parkin-Luciferase-pDrive construct: Luciferase cDNA sequence was amplified by PCR from pGL4.20 [luc2/Puro] vector (Promega) by using primers flanked with *Bgl*II restriction sites on both ends (Operon): forward primer was 5'-GCGAGATCTGAAGATGCCAAAAACATT-3' and reverse primer was 5'-AATAGATCTCACGGCGATCTTGCCG-3'. PCR result was confirmed by DNA gel electrophoresis. The PCR product of luciferase sequence was extracted from agarose gel and eluted by using NucleoSpin® Extract II kit (MACHEREY-NAGEL) according to the manufacturer's recommended procedures. Then, the Fugu parkin-eGFP-pDrive construct was digested with *Bgl*II (New England Biolabs) to remove the eGFP cDNA sequence from the construct; and then eluted luciferase cDNA sequence was inserted into exon 2 of Fugu *parkin* at the *Bgl*II sites of the Fugu parkin-pDrive construct to replace eGFP as the reporter gene. From the Fugu parkin-Luciferase-pDrive construct, I further cloned several Fugu parkin-Luciferase constructs.

CMV-Fugu parkin-Luciferase-pcDNA3.1(-) construct (Figure 7A): the whole Fugu *parkin*-Luciferase sequence containing the full 5'-flanking region of Fugu *Parkin*, obtained from digesting the Fugu parkin-Luciferase-pDrive construct with *Sac*I (New

England Biolabs), was inserted into pcDNA3.1(-) vector with CMV promoter (Invitrogen) at the *Sac*I sites. For cloning the four Fugu parkin-Luciferase constructs containing various deletions of the 5'-flanking region of Fugu *parkin*, restriction enzymes were used to remove the respective fragments of the 5'-flanking region and the CMV promoter from the CMV-Fugu parkin-Luciferase-pcDNA3.1(-) construct (Figure 7A). *SnaB*I, *BspE*I, *BsmB*I, *or Age*I restriction enzymes (New England Biolabs) were used to digest the Fugu parkin-Luciferase-pcDNA3.1(-) construct to remove the CMV promoter and the 5'-flanking region extending from -779 to -687 bp, -779 to -322 bp, -779 to -270 bp, or from -779 to -101 bp respectively; resulting in the 686-Fugu parkin-Luciferase-pcDNA3.1(-) construct (Figure 7B), 321-Fugu parkin-Luciferase-pcDNA3.1(-) construct (Figure 7C), 269-Fugu parkin-Luciferase-pcDNA3.1(-) construct (Figure 7D), or the 100-Fugu parkin-Luciferase-pcDNA3.1(-) construct (Figure 7E), respectively. After enzymatic digestion, Klenow enzyme (Roche) was applied for generating blunt ends, and then the individual plasmid was religated with T4 ligase (New England Biolabs) according to the manufacturer's instruction. CMV-Luciferase-pCMV6-XL5 construct (Figure 7F): the Fugu *parkin*-Luciferase sequence lacking the 5'-flanking region of Fugu *parkin* was obtained by digesting the Fugu parkin-Luciferase-pDrive construct with *Sal*I (New England BioLabs), and was subcloned into pCMV6-XL5 vector with CMV promoter (Origene) at the *Sal*I sites.

Individual plasmids were prepared and purified as described in section 2.2.1a. Plasmid sequences were confirmed by restriction analysis and by DNA sequencing (*StemCore Laboratories, Ottawa Hospital Research Institute).*



Putative nrf2 site in Fugu *parkin* promoter

nrf2: -161 to -151

Forward strand	5′…	CACTTCCGGT …3′
Reverse strand	5′…	ACCGGAAGTG …3′
		::::::::::
Consensus Nrf2		ACCGGAAGNG

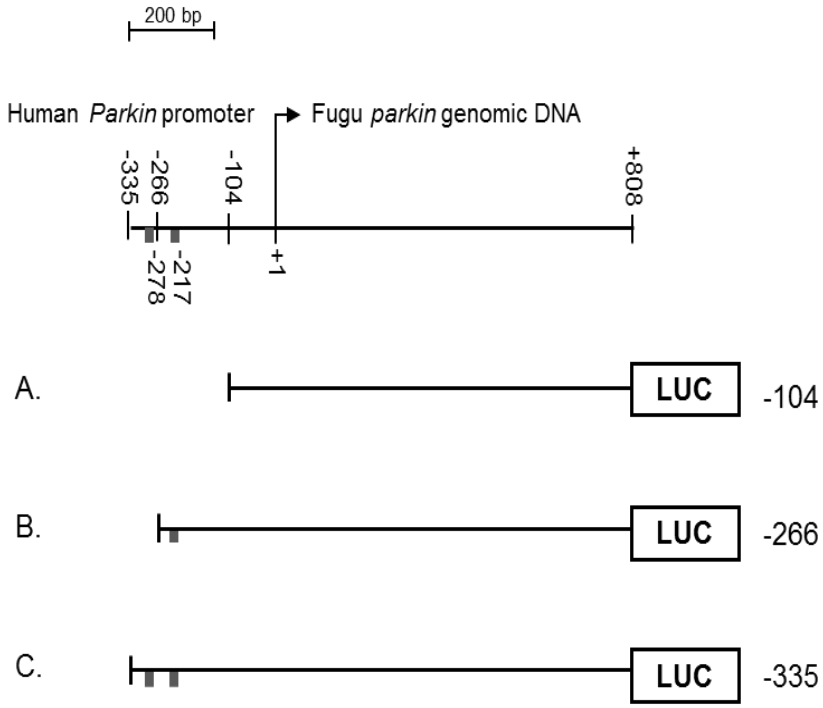
Figure 7. Schema of Fugu parkin-Luciferase constructs. Shown above is the Fugu *parkin* promoter and initial coding region, with sizes designated in bp. The transcription start site (arrow, +1), initiator ATG, and putative Nrf2 element (box) are shown. (A) CMV-Fugu parkin-Luciferase construct containing the full 5'-flanking region with CMV promoter; (B) 686-Fugu parkin-Luciferase-pcDNA3.1(-) construct; (C) 321-Fugu parkin-Luciferase-pcDNA3.1(-) construct; (D) 269-Fugu parkin-Luciferase pcDNA3.1(-) construct; (E) 100-Fugu parkin-Luciferase-pcDNA3.1(-) construct; (F) CMV-Luciferase construct lacking the 5'-flanking region with CMV promoter. Note that in all constructs the luciferase is fused downstream of the initial Fugu *parkin* coding sequence. Promoter map with scale bar is presented schematically with reference to a previous publication (Ou et al., 2000). The sequences comparison was carried out using the sequence alignment program "*ALIGN*" ("*GENESTREAM SEARCH network server IGH Montpellier, France"*) available at "*http://xylian.igh.cnrs.fr/bin/align-guess.cgi*" (Pearson et al., 1997).

2.2.1c Cloning of Human Parkin promoter-Luciferase constructs

Because we were interested in testing whether the putative Nrf2 sites located in the core promoter region of human *Parkin* were involved in *Parkin* expression, I cloned three human Parkin promoter-Luciferase constructs containing various lengths of the 5'-flanking region of human *Parkin* (Figure 8): 104-human Parkin promoter-Luciferase-pcDNA3.1(-) (Figure 8A), 266-human Parkin promoter-Luciferase-pcDNA3.1(-) (Figure 8B), or 335-human Parkin promoter-Luciferase-pcDNA3.1(-) (Figure 8C) constructs were cloned by inserting the nucleotide fragment extending from -104 to +97, -266 to +97, or from -335 to +97, respectively, of the 5'-flanking region of human *Parkin* in front of the ATG start codon of Fugu *parkin* genomic sequence tagged with luciferase cDNA reporter. Thus, as for the Fugu-parkin promoter constructs described above, the same Fugu initial coding sequence is fused to the luciferase. From a construct (provided by Schlossmacher's lab) containing the 5'-flanking region of human *Parkin*, the -104 to +97 bp nucleotide fragment was amplified by PCR using forward primer 5'-CCTTCGCGAGTGAGGAGAAAC-3' and reverse primer 5'-CAAACGCGTGGTCACTGGGTA-3' (Operon); while the -266 to +97 bp nucleotide fragment was amplified by PCR using forward primer 5'-GAATATTCGCGAGGCAGGACCTT-3' and reverse primer 5'-CAAACGCGTGGTCACTGGGTA-3' (Operon); and the -335 to +97 bp nucleotide fragment was amplified by PCR using forward primer 5'-GAATATTCGCGACAGGTTGATCCA-3' and reverse primer 5'-CAAACGCGTGGTCACTGGGTA-3' (Operon). The above primers were flanked with *Nru*I and *Mlu*I restriction sites (Operon) and the individual nucleotide fragment was

inserted immediately upstream of the ATG start codon of Fugu *parkin* by using *Nru*I and *Mlu*I restriction enzymes (New England Biolabs) and then ligated using T4 ligase. Individual plasmids were prepared and purified as described in section 2.2.1a. Plasmid sequences were confirmed by restriction analysis and by DNA sequencing (*StemCore Laboratories* at the *Ottawa Hospital Research Institute).*

According to the "*TRANSFAC*" transcription factor database, "*TFBIND*" and "*TFSEARCH*" softwares, two potential Nrf2 binding sites are located in the core promoter region of human *Parkin*: one potential Nrf2 site (Nrf2(1): "GCCTCCCGGT") spans the -227 to -217 region, while the other potential Nrf2 site (Nrf2(2): "GCGGGAAGAG") spans the -278 to -268 region of human *Parkin* (Figure 8) (Asakawa et al., 2001; Wingender et al., 1996; Heinemeyer et al., 1998; Heinemeyer et al., 1999; Tsunoda and Takagi, 1999; Akiyama, 1995). The -104 to +97 region of human *Parkin* promoter does not contain any consensus Nrf2 binding site, whereas the -266 to +97 region containes one potential Nrf2 binding site: Nrf2(1); while the -335 to +97 region containes both potential Nrf2 binding sites: Nrf2(1) and Nrf2(2) (Figure 8) (Asakawa et al., 2001; Wingender et al., 1996; Heinemeyer et al., 1998; Heinemeyer et al., 1999; Tsunoda and Takagi, 1999; Akiyama, 1995)



Putative Nrf2 sites in human *Parkin* promoter

Nrf2(1): -227 to -217

Forward strand	5′… GCCTCCCGGT …3′
Reverse strand	5′… ACCGGGAGGC …3′
	::::: ::::
Consensus Nrf2	ACCGGAAGNC

Nrf2(2): -278 to -268

Forward strand	5′… GCGGGAAGAG …3′
	: :::::::
Consensus Nrf2	ACCGGAAGNG

Figure 8. Schema of Human Parkin promoter-Luciferase constructs. The following Human Parkin promoter-Luciferase constructs were used in these studies: (A) 104-human Parkin promoter-Luciferase-pcDNA3.1(-), (B) 266-human Parkin promoter-Luciferase-pcDNA3.1(-), (C) 335-human Parkin promoter-Luciferase-pcDNA3.1(-). Two potential Nrf2 sites were located in the human *Parkin* promoter (Asakawa et al., 2001; Wingender et al., 1996; Heinemeyer et al., 1998; Heinemeyer et al., 1999; Tsunoda and Takagi, 1999; Akiyama, 1995). Note that for all constructs the luciferase is fused downstream of the initial Fugu *parkin* coding sequence. Promoter map with scale bar is presented schematically with reference to a previous publication (Ou et al., 2000). The sequences comparisons were carried out using the sequence alignment program "*ALIGN*" ("*GENESTREAM SEARCH network server IGH Montpellier, France"*) available at "*http://xylian.igh.cnrs.fr/bin/align-guess.cgi*" (Pearson et al., 1997).

2.2.2. Site-directed mutagenesis

Since we were interested in testing whether the two potential Nrf2 binding sites in the core promoter region of human *Parkin* are involved in the regulation of *Parkin* expression, I mutated the two putative Nrf2 binding sites by using the site-directed mutagenesis protocol ("*Kyle Gurley version 1.0*") (Gurley, 2004). Briefly, the method required four primers and three PCR reactions; in which two primers were designed to target the putative Nrf2 site, while the other two primers targeted the 5'-flanking region and 3'-flanking region of *parkin* gene (Gurley, 2004).

The following primers (Sigma) were used for site-directed mutagenesis to mutate the putative Nrf2(1) binding site "GCCTCCCGGT" to "GCCTTTTGGT":

F1: 5'-GCTGCAACAAGCTTCCAAAGGTAAGCCTTTTGGTTGCTAAGCGACT-3'

R1: 5'-AGTCGCTTAGCAACCAAAAGGCTTACCTTTGGAAGCTTGTTGCAGC-3'

The following primers (Sigma) were used to mutate the putative Nrf2(2) binding site "GCGGGAAGAG" to "GCAAAAAGAG":

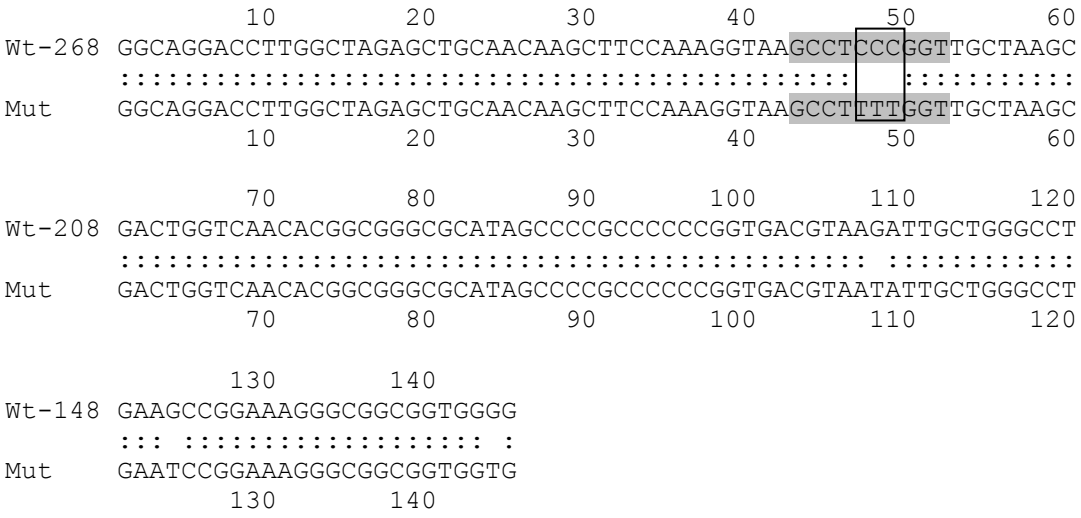
F2: 5'-CCCTAGGGGCGGGGCAAAAAGAGGGCAGGACCT-3'

R2: 5'-AGGTCCTGCCCTCTTTTTGCCCCGCCCCTAGGG-3'.

The sequence of the mutant constructs was confirmed by automated sequence analysis (*StemCore Laboratories, Ottawa Hospital Research Institute).* Then, the sequence of the human *Parkin* promoter containing wild-type Nrf2 binding site was compared with the sequence of human *Parkin* promoter with site-directed mutagenesis obtained from DNA sequencing (Asakawa et al., 2001). The sequences comparisons were carried out using

the sequence alignment program "*ALIGN*" ("*GENESTREAM SEARCH network server IGH Montpellier, France"*) available at "*http://xylian.igh.cnrs.fr/bin/align-guess.cgi*" (Pearson et al., 1997). As shown in Figure 9, the putative Nrf2(1) binding sequence spanning the -227 to -217 of human *Parkin* promoter was successfully mutated from "GCCTCCCGGT" to "GCCTTTTGGT"; while the putative Nrf2(2) binding sequence spanning the -278 to -268 of human *Parkin* promoter was successfully mutated from "GCGGGAAGAG" to "GCAAAAAGAG".

Nrf2(1) site: GCCTCCCGGT



Nrf2(2) site: GCGGGAAGAG

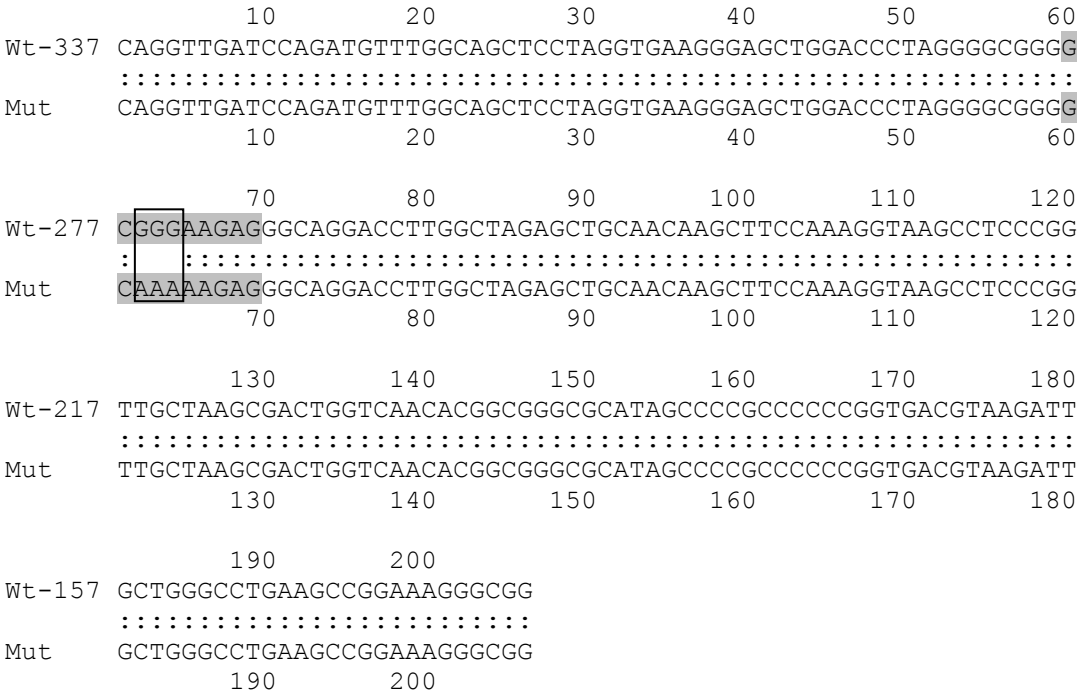


Figure 9. Nucleotide sequences comparisons of human *Parkin* promoters with wild-type or mutated Nrf2 binding sites. DNA sequencing reactions were processed and carried out by the *StemCore Laboratories* at the *Ottawa Hospital Research Institute.* DNA sequencing results was used to determine if the two targeted potential Nrf2 binding sequences were successfully mutated (Asakawa et al., 2001; Gurley, 2004). The sequences were aligned and compared by using the sequence alignment program "*ALIGN*" ("*GENESTREAM SEARCH network server IGH Montpellier, France"*) available at "*http://xylian.igh.cnrs.fr/bin/align-guess.cgi"* (Pearson et al., 1997).

2.2.3 Mammalian cell culture and transient transfection

Since a previous study had demonstrated that certain cell lines including the Chinese Hamster Ovary (CHO) (ATCC), human brain neuroblastoma SH-SY5Y (ATCC), and human kidney HEK (Human Embryonic Kidney)-293 (ATCC) express endogenous Parkin protein (Pawlyk et al., 2003), we considered these cell lines to be suitable for studying regulation of *Parkin* expression. CHO cells and HEK-293 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. SH-SY5Y cells were cultured in 1:1 mixture of D-MEM and Ham's F12 medium, supplemented with 10% FBS, 1% Penicillin/Streptomycin, 0.5% Sodium Pyruvate (100 mM) and 0.75% L-glutamine. Cells were incubated in a humidified 5% carbon dioxide (CO_2) atmosphere at 37℃. For cell transfection, cells were cultured in growth medium without antibiotics. Lipofectamine 2000 (Invitrogen) was used as transfection reagent and cell transfection was performed according to the manufacturer's recommended procedures.

2.2.4 Drug treatment

Cell cultures were treated with different concentrations of various chemicals obtained from Sigma: L-buthionine-sulfoximine (BSO) (purity ≥ 97%), diethyl maleate (DEM) (purity 97 %), *tert*-Butylhydroquinone (tBHQ) (purity 97%), or copper (II) chloride ($CuCl_2$) (purity 97%). For preparing the 0.1 M stock solution for cell treatment, BSO, $CuCl_2$, DEM, or tBHQ was dissolved in dimethyl sulfoxide (DMSO) (purity ≥ 99.9%)

(Sigma). The working solution of BSO, $CuCl_2$, DEM or tBHQ was prepared by diluting the stock solution with Dulbecco's Modified Eagle's Medium with DMSO at a final concentration of 0.05 %. DMSO (0.05%) was used as vehicle control for drug treatment.

2.2.5 Fluorescence microscopy

After cell transfection, the cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) and ProLong® Gold antifade reagent (Invitrogen) was used for preserving the signals. EGFP expression in the cells was monitored under fluorescence microscope and pictures were taken at the same time (Zeiss Axioplan, AxioCamICc3, 40X magnification).

2.2.6 Dual-Luciferase Reporter Assay

The relative luciferase activity (transcription activity) of Fugu parkin-Luciferase constructs or Human Parkin promoter-Luciferase constructs were measured by performing the Dual-Luciferase Reporter Assay (Promega) according to the manufacturer's recommended procedures. Briefly, cells seeded in 12-well plates were co-transfected with Fugu parkin-Luciferase construct or Human Parkin promoter-Luciferase construct (containing the firefly luciferase sequence) and pRL CMV-Renilla luciferase construct ("*internal vector control*") (Promega). For detecting the relative luciferase activity, transfected cells in 12-well plates were lysed by 1X "*passive lysis buffer*" provided in the Dual-Luciferase Reporter Assay kit (Promega), and gently vibrated at

room temperature for 15 minutes according to the manufacture's instruction (Promega). Cell lysate was collected and 25 µl of cell lysate was loaded per well of 96-well plate for analysis. By using a luminometer with reagent auto-injectors (LUMIstar OPTIMA, or Promega), Luciferase Assay Reagent II (Promega) was added to the sample for measuring the firefly luciferase activity, then the Stop & Glo® Reagent (Promega) was added to the same sample for measuring the *Renilla* luciferase activity. For normalization of cell transfection efficiency, the reading of firefly luciferase was divided by that of *Renilla* luciferase (Promega).

2.2.7 Western Blotting

Western blot analysis was performed as described previously with modifications (Ferguson et al., 2002; Wong 2014; Schlossmacher et al., 2002; Cullen et al. 2009): Briefly, transfected cells were collected and lysed with ice-cold cell lysis buffer (50 mM Tris pH7.5, 150 mM NaCl, 1% Triton X-100, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS (Sodium dodecyl sulfate), 2 mM EDTA). Cell lysate was centrifuged at 13,000 g for 15 minutes at 4 ℃; and then supernatant containing the total cell protein was collected. BCA (Bicinchoninic acid assay) protein assay (Thermo Scientific) was used to determine the protein concentration of cell lysate. Appropriate concentration of cell lysate was mixed with SDS loading buffer, and boiled at 95 ℃ for 5 minutes. The total protein samples were separated by SDS PAGE (polyacrylamide gel electrophoresis) gel: 10% Separating Gel (0.5 M Tris, 1.5 M Glycine (pH8.8), 0.4% SDS, 5% glycerol, 10% acrylamide, 0.0625% bisacrylamide, 0.1% ammonium persulfate and 0.1%

Tetramethylethylenediamine (TEMED) in double-distilled water) and 4% Stacking gel: 0.5 M Tris-HCL (Tris-hydrochloride) (pH6.8), 0.4% SDS, 2.5% glycerol, 50 mM Ethylenediaminetetraacetic acid (EDTA) (pH 7.0), 5% acrylamide, 0.1% ammonium persulfate and 0.1% TEMED in double-distilled water) (Alternatively, NuPAGE 4-12% Bis-Tris Pre-Cast gels (Invitrogen) were used). Precision Plus Protein™ Prestained Standard (Bio-Rad) was used to determine the molecular weight of the separated protein bands. After electrophoresis, proteins were transferred onto 0.45-micron polyvinyl difluoride (PVDF) membranes. Then, PVDF membranes were blocked in Phosphate Buffered Saline containing Tween 20 (PBST) in 5% skim milk for 1 hour in room temperature. After that, PVDF membranes were rinsed with PBST to remove excessive blocking buffer; appropriate antibody diluted in PBST with 1-5% milk was added to the membrane for detecting the targeted protein. EGFP protein or Fugu parkin-eGFP protein were detected using anti-GFP antibody (Clontech) at 1:2000 dilution and incubated for 2 hours at room temperature. Parkin protein was detected by using anti-parkin (PRK8) antibody (Millipore or Sigma) (1:1000, overnight at 4 ℃). Myc-Nrf2 protein was detected by using anti-Myc-Tag (9B11) antibody (Cell Signaling) (1:1000, overnight at 4℃). HO-1 protein was detected by anti-Heme Oxygenase 1 antibody (Abcam) (1:250, overnight at 4℃). Keap1 protein was detected by anti-Keap1 antibody (Abcam) (1:1000, overnight at 4℃). Beta actin (β-actin) was used for loading control of Western blot analysis, and it was detected by anti-beta-actin antibody (Abcam) (1:5000, 2 hour at room temperature or overnight at 4℃). Horseradish Peroxidase (HRP)-Conjugated anti-mouse secondary antibody or HRP-Conjugated anti-rabbit secondary antibody in PBST with 1-5% skim milk (1:10000, 1 hour at room temperature) was used to detect the antigen-

primary antibody complex. ImageJ (National Institutes of Health) was used to measure the densitometry of protein bands; and relative amounts of Parkin protein, Nrf2 protein, Keap1 protein, HO-1 protein were calculated after normalization to β-actin protein on the same sample.

2.2.8 Isolation and culture of primary neurons

A protocol for the isolation of mouse embryonic primary neurons was provided by Dr. David Park's laboratory. Primary neurons were isolated from wild type mice (C57BL/6) at 14-15 days gestation and cultured as described previously (Brewer et al., 1993; Xiang et al., 1996). Primary neurons were cultured in complete Neurobasal Medium with 2% B27 Supplement, 1% N2 Supplement, 0.5% Penicillin/Streptomycin and 0.025% L-glutamine; and were incubated in a humidified 5% CO_2 atmosphere at 37℃ (Brewer et al., 1993; Xiang et al., 1996).

2.2.9 Data analysis

Data were analyzed using the SPSS software. One-way analysis of variance (ANOVA) followed by Dunnett's post-test was used to compare the tested groups with the control. T-test was used for comparing two groups. P value less than 0.05 was considered as statistically significant and is marked as *, while p value less than 0.01 was marked as **.

ImageJ (National Institutes of Health) was used to quantify bands from Western Blot images.

CHAPTER III

RESULTS

3.1 Validation of Fugu parkin and human Parkin expression in CHO cells

In order to study the cis- and trans-acting transcription factors involved in *Parkin* gene expression, we wanted to establish a suitable cell model. Since previous study showed that CHO (Chinese Hamster Ovary) cells express endogenous parkin (Pawlyk et al., 2003), and parkin expression in CHO cells has been verified in Dr. Schlossmacher's laboratory, we considered CHO cells to be suitable for this study. Moreover, we also wanted to validate the expression of exogenous human Parkin and Fugu parkin in CHO cells following transfection. Since the anti-Parkin antibody cannot detect Fugu parkin protein, Yu *et al.* cloned the Flag-tagged Fugu parkin construct and the Flag-tagged human Parkin construct in which a Flag-tag was cloned in frame at the N-terminal of *parkin* so that the Flag-tagged Fugu parkin can be detected by anti-Flag antibody (Yu et al., 2005). Yu *et al.* successfully detected the expression of Flag-tagged Fugu parkin protein with a molecular weight (MW) of 55 kDa (Kilodaltons) and Flag-tagged human Parkin protein (52 kDa) in transfected HEK293 cells (Yu et al., 2005). The Flag-tagged Fugu parkin construct contained the 11 exons of Fugu *parkin* (Yu et al., 2005). In the current study, CHO cells were transfected with Flag-tagged Fugu parkin construct or Flag-tagged human Parkin construct (Yu et al., 2005), or with empty vector as negative control. As shown in Figure 10, both Flag-tagged Fugu parkin (55 kDa) (Lane 2) and Flag-tagged human Parkin (52 kDa) (Lane 3) were detected in CHO cells by using the anti-Flag antibody. No parkin expression was detected in negative controls (Lane 1), suggesting that exogenous Fugu parkin and human Parkin are expressed following transfection in CHO cells.

Figure 10.

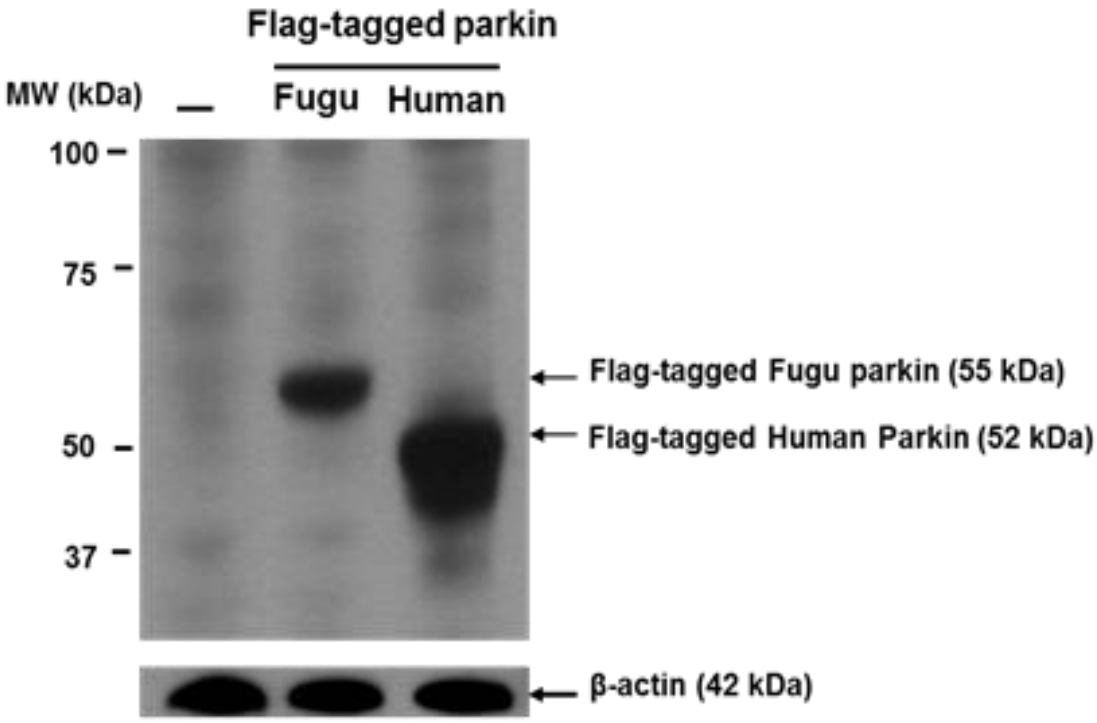

Flag-tagged parkin
MW (kDa)
—
Fugu
Human
100
75
50
37
Flag-tagged Fugu parkin (55 kDa)
Flag-tagged Human Parkin (52 kDa)
β-actin (42 kDa)

Figure 10. Validation of cell transfection and parkin expression

Detection of Flag-tagged Fugu Parkin and Flag-tagged human Parkin expression by Western blotting. Lane 1: CHO cells were transfected with empty vector as negative control. Lane 2: CHO cells were transfected with Flag-tagged Fugu parkin construct (Yu et al., 2005). Lane 3: CHO cells were transfected with Flag-tagged human Parkin construct (Yu *et al.*, 2005). 24 hours after cell transfection, cells were lysed with cell lysis buffer for Western blot analysis. Anti-Flag antibody (1:1000) (Sigma) was used to detect Flag-tagged Fugu parkin protein and Flag-tagged human Parkin protein, and anti-β-actin antibody (1:5000) (Abcam) was used to detect β-actin protein.

Experiments were performed again with similar results: For detection of Flag-tagged Fugu parkin, CHO cells were transfected with various amounts of Flag-tagged Fugu parkin constructs and detected by anti-Flag antibody. For detection of Flag-tagged human Parkin, CHO cells were transfected with Flag-tagged human Parkin constructs and detected by anti-Parkin antibody.

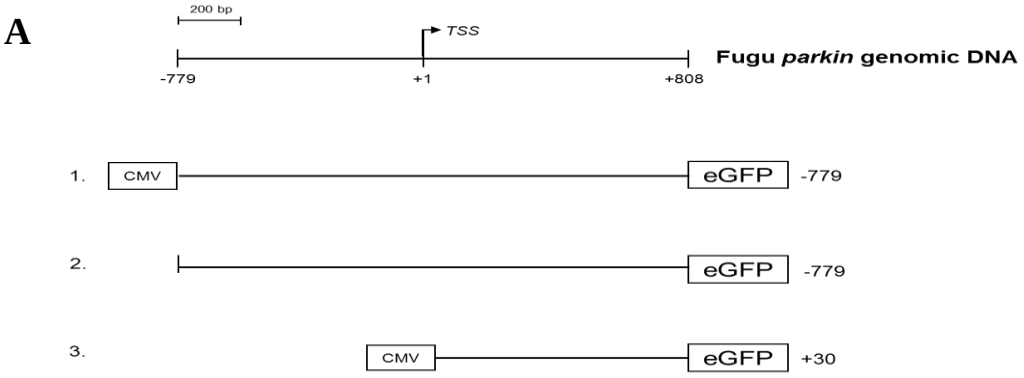
3.2 Expression of Fugu-parkin-eGFP in CHO cells

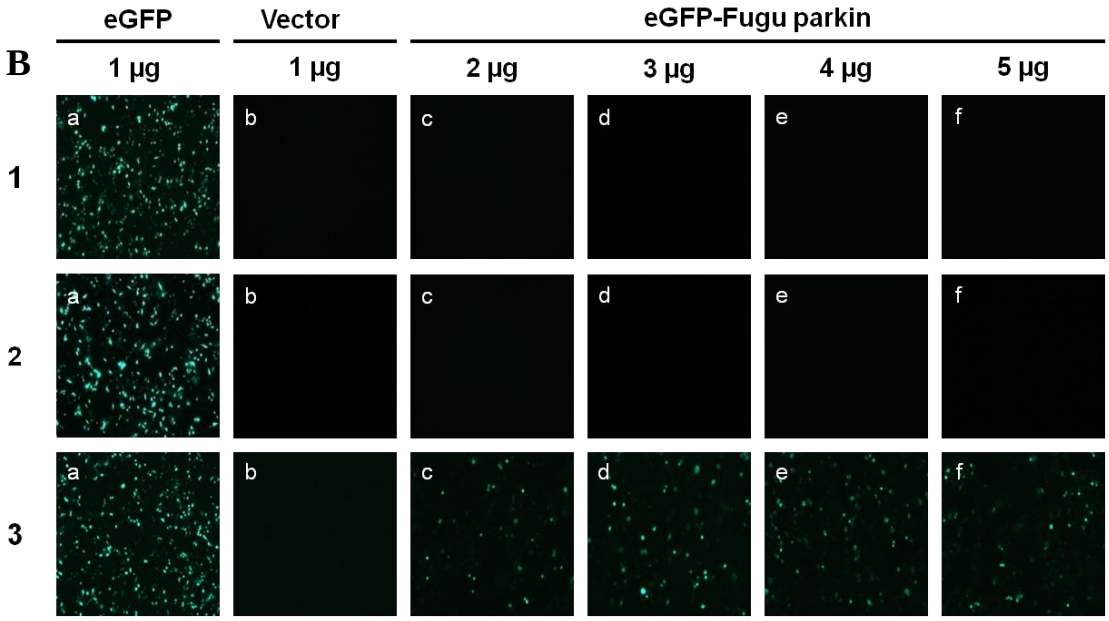
In order to test if *parkin* promoter activity could be detected in CHO cells using eGFP in the form of fusion proteins as a readout, CHO cells were transfected with various concentrations (1 µg (microgram) to 5 µg) of CMV-Fugu parkin-eGFP construct containing full 5'-flanking region of Fugu *parkin* with the CMV promoter (Figure 11A, construct 1), or with Fugu parkin-eGFP construct containing full Fugu *parkin* 5'-flanking region without the CMV promoter (Figure 11A, construct 2), or with CMV-eGFP-pCMV6-XL5 construct lacking the 5'-flanking region with the CMV promoter (Figure 11A, construct 3). CHO cells transfected with the empty pcDNA3.1(-) or pCMV6-XL5 vector served as negative control for eGFP expression, while CHO cells transfected with eGFP plasmid served as positive control. Fluorescence microscopy was used to detect the eGFP signal, while Western blotting was used to detect eGFP protein expression.

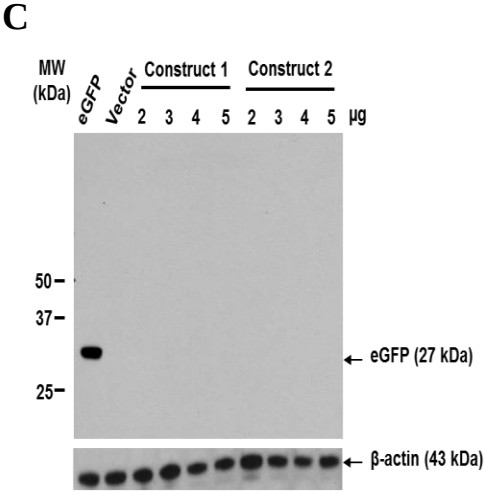
As shown in Figure 11B, the results of fluorescence microscopy showed that an eGFP signal was detected in CHO cells transfected with the eGFP construct (a), whereas no eGFP signal was detected in CHO cells transfected with empty vector (b), suggesting that the cell transfection and detection procedures were correct. There was no eGFP signal detected in CHO cells transfected with eGFP constructs containing the full 5'-flanking region of Fugu *parkin* (with or without CMV promoter) whereas an eGFP signal was detected in CHO cells transfected with 2 µg to 5 µg of CMV-eGFP construct lacking the 5'-flanking region of Fugu *parkin* (c-f). This result indicated that the Fugu promoter was preventing expression from the CMV promoter in mammalian CHO cells, while the CMV promoter alone was able to drive expression of Fugu parkin-GFP (construct 3).

As shown in Figure 11C, Western blot analysis results agreed with the results from fluorescence microscopy, in that eGFP protein (27-kDa) was detected in CHO cells transfected with eGFP construct (Lane 1). Since no eGFP was detected in CHO cells transfected with empty pcDNA3.1(-) vector (Lane 2), we considered the eGFP detected by the anti-GFP antibody to be specific. No Fugu parkin-eGFP fusion protein (35 kDa) was detected in CHO cells transfected with Fugu parkin-eGFP construct containing the full 5'-flanking region of Fugu *parkin* (Lanes 3-10 of Figure 11C); whereas Fugu parkin-eGFP fusion protein (35 kDa) was detected in CHO cells transfected with 1 μg to 4 μg of CMV-eGFP construct lacking the 5'-flanking region (Lanes 3-6 of Figure 11D). β-actin (42 kDa) was detected in all lanes as a loading control. The proportion of GFP-stained cells or GFP-tagged Parkin did not increase with increasing amounts of construct 3, thereby suggesting that maximal transfection had been obtained.

Because no expression of Fugu parkin-eGFP was detected even in the presence of the strong promoter (CMV promoter), the results of fluorescence microscopy and Western blot suggested that the Fugu *parkin* might contain a strong repressive element in the 5'-flanking region that inhibited transcription.


A
200 bp
TSS
-779
+1
+808
Fugu parkin genomic DNA
1.
CMV
eGFP
-779
2.
eGFP
-779
3.
CMV
eGFP
+30


B
eGFP
Vector
eGFP-Fugu parkin
1 μg
1 μg
2 μg
3 μg
4 μg
5 μg
1
2
3
a
b
c
d
e
f


C
MW (kDa)
eGFP
Vector
Construct 1
Construct 2
2 3 4 5 2 3 4 5 μg
50
37
25
eGFP (27 kDa)
β-actin (43 kDa)

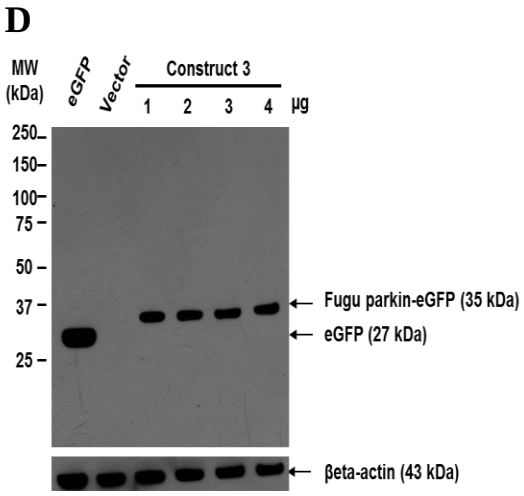

D
MW (kDa)
eGFP
Vector
Construct 3
1 2 3 4 μg
250
150
100
75
50
37
25
Fugu parkin-eGFP (35 kDa)
eGFP (27 kDa)
βeta-actin (43 kDa)

Figure 11. CHO cells transfection with Fugu parkin-eGFP constructs. (A) Construct 1: CMV-Fugu parkin-eGFP construct containing full 5'-flanking region of Fugu *parkin* with CMV promoter; Construct 2: Fugu parkin-eGFP construct containing full 5'-flanking region without CMV promoter; Construct 3: CMV-eGFP construct lacking the 5'-flanking region with CMV promoter. (B) Detection of eGFP signal by fluorescence microscopy. (a) CHO cells were transected with 1 μg of eGFP plasmid (positive control). (b) CHO cells were transfected with 1 μg of pcDNA3.1(-) or pCMV6-XL5 empty vector (negative control). (c-f), CHO cells were transfected with 2 μg, 3 μg, 4 μg, or 5 μg of Fugu parkin-eGFP construct. Column 1: Construct 1; Column 2: Construct 2; Column 3: Construct 3. (C) Detection of Fugu parkin-eGFP expression by Western blotting. Lane 1: CHO cells were transfected with 1 μg of eGFP plasmid (positive control). Lane 2: CHO cells were transfected with 1 μg of pcDNA3.1(-) empty vector (negative control). Lanes 3-6: CHO cells were transfected with 2 μg - 5 μg of construct 1 (with full 5' and CMV). Lanes 7-10, CHO cells were transfected with 2 μg - 5 μg of construct 2 (with full 5'). (D) Detection of eGFP expression by Western blotting. Lane 1: CHO cells were transfected with 1 μg of eGFP plasmid (positive control). Lane 2: CHO cells were transfected with 1 μg of pCMV6-XL5 empty vector (negative control). Lanes 3-6: CHO cells were transfected with 1 μg - 4 μg of construct 3 (lacking 5'). 24 hours after cell transfection, cells were lysed with cell lysis buffer for Western blot analysis. Anti-GFP antibody (1:2000) (Clontech) was used to detect eGFP and Fugu parkin-eGFP fusion protein, and anti-β-actin antibody (1:5000) (Abcam) was used to detect β-actin as a loading control. Experiments (D) were performed three times with similar results.

3.3 Transcriptional activity of various Fugu *parkin* promoter regions

3.3.1 CHO cells transfection with Fugu parkin-Luciferase constructs with or without 5'-flanking region

Since the experimental results from the eGFP-Fugu parkin constructs suggested that the 5'-flanking region of Fugu *parkin* might contain repressive element that hindering the expression of Fugu *parkin*, we validated these results by comparing the relative luciferase activity of the CMV-Fugu parkin-Luciferase construct containing the full 5'-flanking region and the CMV-Luciferase construct lacking the 5'-flanking region with CMV promoter in CHO cells. For each construct, CHO cells were co-transfected with 5 ng (nanogram) of the pRL CMV Renilla luciferase construct (Promega) to normalize transfection efficiency. As shown in Figure 12, Luciferase-Fugu *parkin* construct lacking the 5'-flanking region of Fugu *parkin* had significantly higher luciferase activity ($p<0.05$; $p=0.016$) than the Luciferase-Fugu *parkin* construct containing the entire 5'-flanking region of Fugu *parkin*. This result suggested that the presence of the entire 5'-flanking region of Fugu *parkin* might repress the transcriptional activity of Fugu *parkin*.

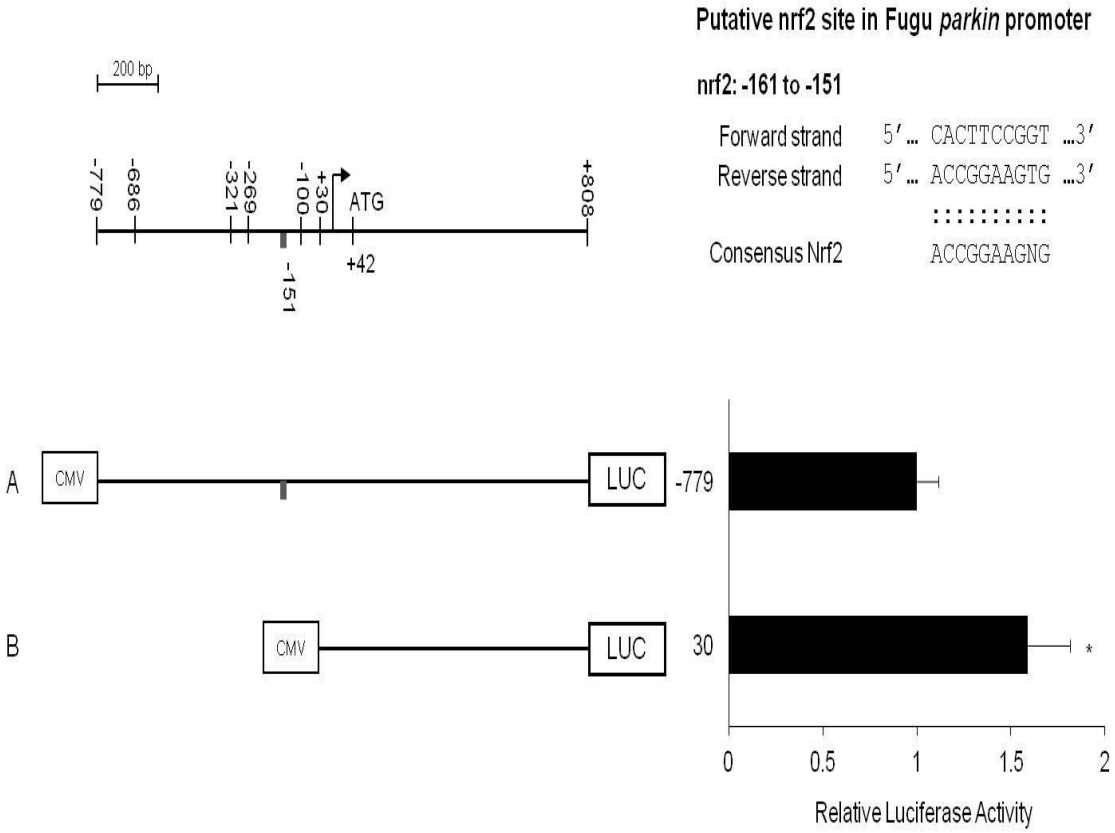
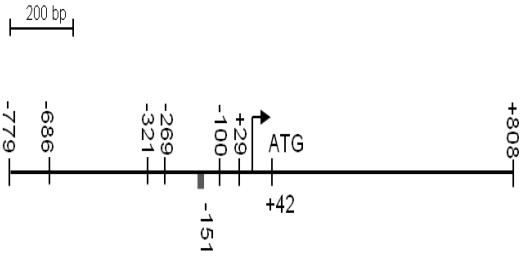

Putative nrf2 site in Fugu *parkin* promoter
nrf2: -161 to -151
Forward strand 5′... CACTTCCGGT ...3′
Reverse strand 5′... ACCGGAAGTG ...3′
Consensus Nrf2 ACCGGAAGNG
200 bp
-779
-686
-321
-269
-100
+30
ATG
+808
-151
+42
A
CMV
LUC
-779
B
CMV
LUC
30
*
0
0.5
1
1.5
2
Relative Luciferase Activity

Figure 12. Relative luciferase activity of Fugu *parkin* with or without 5'-flanking region. Shown above and at left is a schematic representation of the CMV promoter (CMV) fused to Fugu Parkin gene luciferase constructs with promoter, transcription (+1 bp) and translation start (ATG, +42 bp) and luciferase (LUC); the black bar represents the potential Nrf2 site (sequence shown at right). CHO cells were transfected with the CMV-Fugu parkin-Luciferase construct containing the full 5'-flanking region (A), or with the CMV-Luciferase construct lacking the 5'-flanking region with CMV promoter in CHO cells. For each construct, CHO cells were co-transfected with 5 ng pRL CMV Renilla luciferase construct (Promega) for normalization of cell transfection efficiency. Cells were lysed for measurement of luciferase activity by the Dual-Luciferase Reporter Assay 24 hours after transfection (Promega). Relative luciferase activity of the constructs was normalized to Renilla luciferase activity; and then the results were normalized to the mean value for the CMV-Fugu parkin-Luciferase construct containing the full 5'-flanking region (construct A). In this way, the normalized relative luciferase activity of construct A was 1, and the normalized relative luciferase activity of constructs was relative to that of construct A. T-test was used for comparing the two groups. The result is shown as mean ± SEM (standard error of the mean). Experiments were performed in triplicate.

3.3.2 CHO cell transfection with Fugu parkin-Luciferase with truncated 5'-flanking region

The previous result suggested that Fugu-parkin promoter may repress or prevent CMV-driven transcription. In order to determine whether the Fugu-parkin promoter has activity in CHO cells, the promoter activity of various lengths of the 5'-flanking region of Fugu *parkin* without the CMV promoter was tested. CHO cells were transfected with 1 μg of the 686-Fugu parkin-Luciferase constructs, 321-Fugu parkin-Luciferase constructs, 269-Fugu parkin-Luciferase constructs, or 100-Fugu parkin-Luciferase constructs (Figure 13). CHO cells transfected with 100-Fugu parkin-Luciferase constructs served as baseline for minimal Fugu *parkin* promoter activity. For each construct, CHO cells were co-transfected with 5 ng of the pRL CMV Renilla luciferase construct (Promega). As shown in Figure 13, when compared with the 100-Fugu parkin-Luciferase construct (control), the 269-Fugu parkin-Luciferase construct has significantly higher luciferase activity ($p<0.05$), suggesting that a weak enhancer region extending from -269 to -100 bp might be important for the transcription activity of Fugu *parkin*. In contrast, the 686- and 321-Fugu parkin constructs did not differ from the 100-Fugu parkin construct suggesting that an upstream repressor is located between -321 and -269 bp. Interestingly, the most active portion of the promoter region extending from -269 to -100 contains one potential Nrf2 binding site.



Putative nrf2 site in Fugu *parkin* promoter

nrf2: -161 to -151

Forward strand	5′... CACTTCCGGT ...3′
Reverse strand	5′... ACCGGAAGTG ...3′
	::::::::::
Consensus Nrf2	ACCGGAAGNG

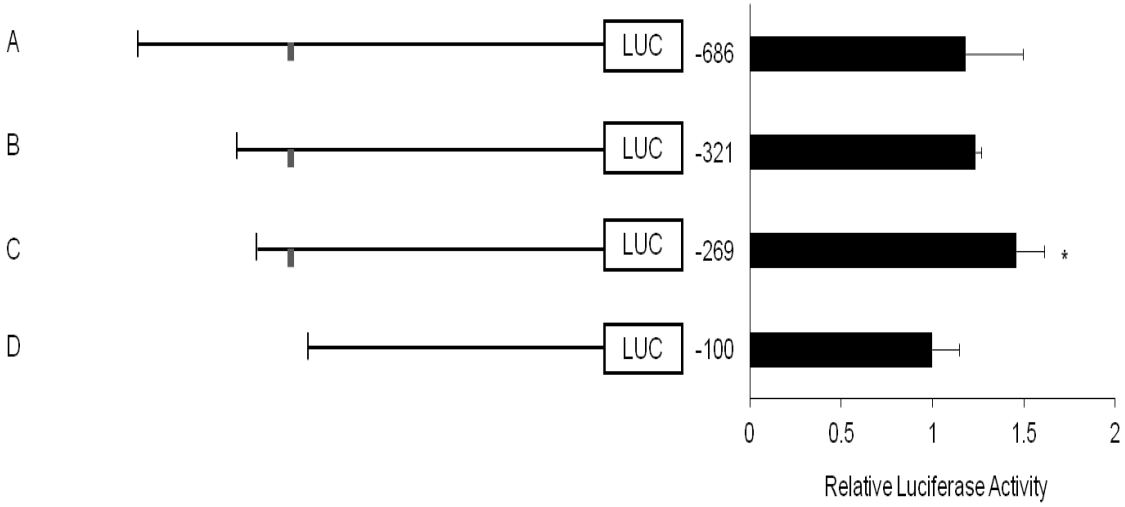


Figure 13. Relative luciferase activity of various promoter fragments of Fugu *parkin*. Shown above and at left is a schematic representation of the Fugu Parkin gene luciferase constructs with promoter, transcription (+1 bp) and translation start (ATG, +42 bp) and luciferase (LUC); the black bar represents the potential Nrf2 site (sequence shown at right). CHO cells were transfected with 1 μg of the 686-Fugu parkin-Luciferase construct (A), or the 321-Fugu parkin-Luciferase construct (B), or the 269-Fugu parkin-Luciferase construct (C), or the 100-Fugu parkin-Luciferase construct (D). For each construct, CHO cells were co-transfected with 5 ng pRL CMV Renilla luciferase construct (Promega) for normalization of cell transfection efficiency. 24 hours after transfection, cells were lysed for measurement of luciferase activity by the Dual-Luciferase Reporter Assay (Promega). Relative luciferase activity of the constructs was normalized to Renilla luciferase activity; and the results were normalized to the mean value of the 100-Fugu parkin-Luciferase construct (control). One-way analysis of variance (ANOVA) followed by Dunnett's post-test was used to compare the tested groups with the control. The result is shown as mean ± SEM (standard error of the mean). Experiments were performed in triplicate.

3.4 Effect of Nrf2 pathway activators on parkin protein expression

3.4.1 Validation of transcriptional activity of Fugu parkin-Luciferase in CHO cells

3.4.1a CHO cells transfection with 269-Fugu parkin-Luciferase-pcDNA3.1(-) construct

Because we wanted to utilize the 269-Fugu parkin-Luciferase-pcDNA3.1(-) construct to study the effect of potential cis- and trans- transcription factors involved in *parkin* expression, we tested different amounts of 269-Fugu parkin-Luciferase-pcDNA3.1(-) construct for cell transfection. An optimal amount would allow us to detect the basal transcriptional activity of *parkin* without treatment, and would allow us to detect any increase or decrease in the transcriptional acitivity *of parkin* with treatment. As shown in Figure 14A, significantly higher luciferase activity was detected in CHO cells transfected with 500 ng ($p<0.05$) or 1000 ng ($p<0.01$) of Fugu parkin-Luciferase construct compared to pcDNA3.1(-) vector. Similar level of luciferase activities were detected in CHO cells transfected with 62.5 ng, 125 ng, and 250 ng of Fugu parkin-Luciferase; and their relative luciferase activities were not significantly higher than the pcDNA3.1(-) empty vector. This experimental result indicates that the -269/+41 bp fragment of Fugu *parkin* has significant promoter activity compared to vector, and suggested that the transcriptional activity of Fugu parkin-Luciferase driven by the Fugu *parkin* promoter sequence could be monitored in CHO cells. Moreover, this result suggested that 62.5 ng to 250 ng of 269-Fugu parkin-Luciferase construct might be appropriate for studying the effects of cis- and trans- transcription factor for activation of *parkin* transcription; for suppression of transcription, 1000 ng would be optimal.

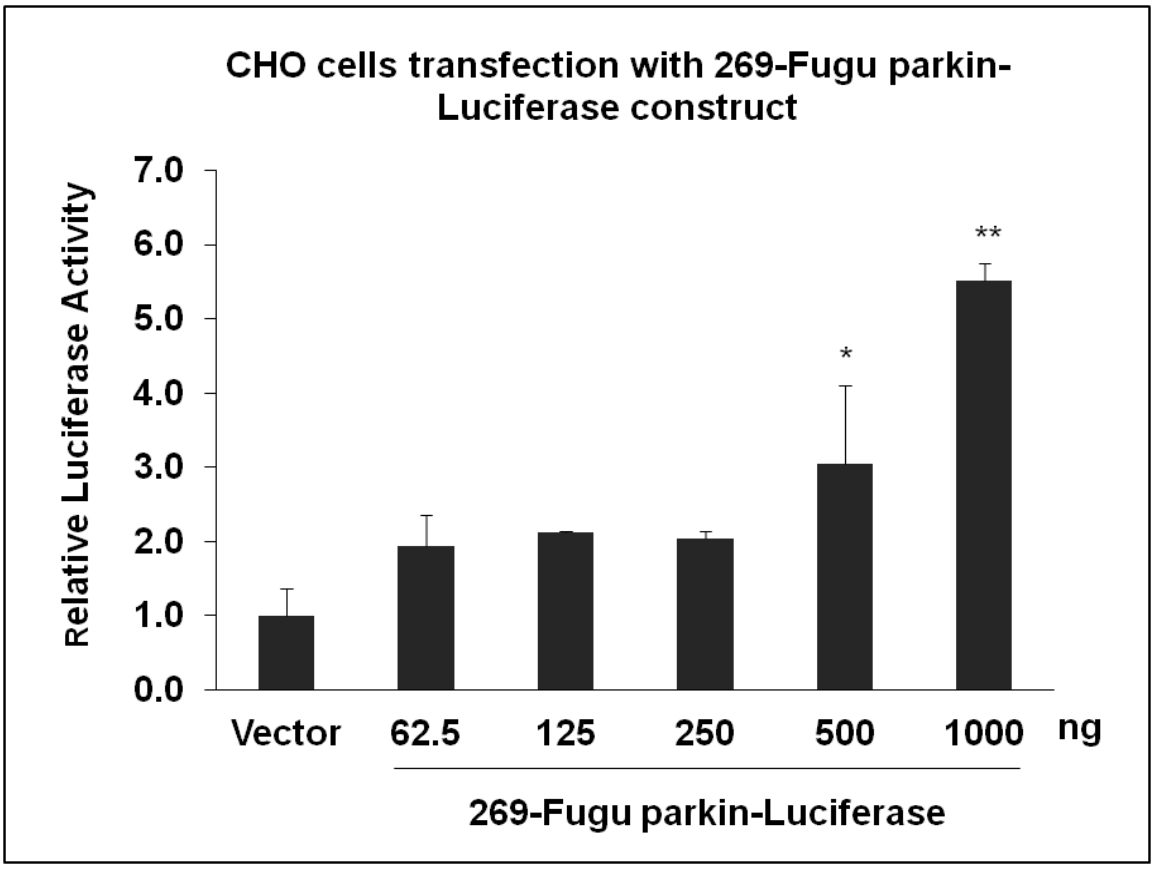

CHO cells transfection with 269-Fugu parkin-Luciferase construct
Relative Luciferase Activity
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
*
**
Vector
62.5
125
250
500
1000
ng
269-Fugu parkin-Luciferase

Figure 14A. CHO cells transfection with increasing amounts of 269-Fugu parkin-Luciferase construct. Bar 1: CHO cells were transfected with pcDNA3.1 (-) empty vector (negative control). Bars 2-6: CHO cells were transfected with 62.5 ng, 125 ng, 250 ng, 500 ng, 1000 ng of 269-Fugu parkin-Luciferase-pcDNA3.1 (-) construct, respectively. For each transfection, empty vector was added so that the total aount of construct was 1000 ng for each transfection. For each transfection, CHO cells were co-transfected with 5 ng pRL CMV Renilla luciferase construct (Promega). 24 hours after cell transfection, cells were lysed and luciferase activity was measured by performing the Dual-Luciferase Reporter Assay (Promega) according to the manufacturer's recommended procedures. Relative luciferase activity of Luciferase-Fugu parkin was normalized by Renilla luciferase activities and normalized to the empty vector control (1.0), and is shown as mean ± SEM. Experiments were performed in triplicate. One-way analysis of variance (ANOVA) followed by Dunnett's post-test was used to compare the tested groups with the control.

3.4.1b CHO cells transfection with 104-human Parkin promoter-Luciferase-pcDNA3.1(-)

In addition, we have tested the activity of different amounts of the construct termed "104-human Parkin promoter-Luciferase-pcDNA3.1(-)" for cell transfection. As shown in Fig. 14B, significantly higher luciferase activity was detected in CHO cells transfected with 125 ng ($p<0.05$), 250 ng ($p<0.05$), 500 ng ($p<0.01$) or 1000 ng ($p<0.01$) of 104-human Parkin promoter-Luciferase construct compared to vector. This experimental result suggested that the transcriptional activity of Fugu parkin-Luciferase driven by human *Parkin* promoter region could be monitored in CHO cells. Moreover, this result suggested that 125-250 ng of 104-human Parkin promoter-Luciferase construct might be appropriate for studying the effects of cis- and trans- transcription factor on *parkin* transcriptional activity.

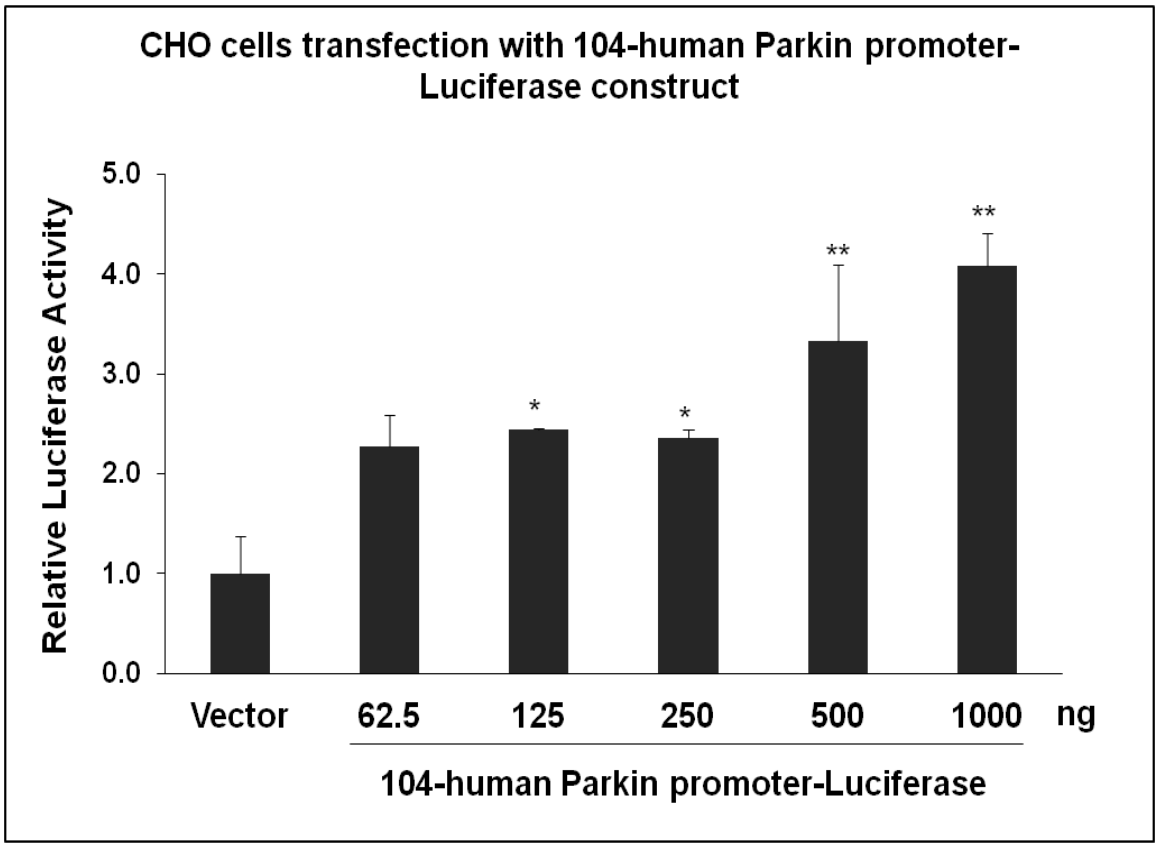

CHO cells transfection with 104-human Parkin promoter-Luciferase construct
Relative Luciferase Activity
5.0
4.0
3.0
2.0
1.0
0.0
*
*
**
**
Vector
62.5
125
250
500
1000
ng
104-human Parkin promoter-Luciferase

Figure 14B. CHO cells transfection with 104-human Parkin promoter-Luciferase-pcDNA3.1(-). Bar 1: CHO cells were transfected with 1000 ng of pcDNA3.1 (-) empty vector (negative control). Bars 2-6: CHO cells were transfected with 62.5 ng, 125 ng, 250 ng, 500 ng, 1000 ng of 104-human Parkin promoter-Luciferase-pcDNA3.1(-) construct, respectively. For each transfection, empty vector was added so that the total aount of construct was 1000 ng for each transfection. For each transfection, CHO cells were co-transfected with 5 ng pRL CMV Renilla luciferase construct (Promega). 24 hours after cell transfection, cells were lysed and luciferase activity was measured by performing the Dual-Luciferase Reporter Assay (Promega) according to the manufacturer's recommended procedures. Relative luciferase activity of Luciferase-Fugu parkin was normalized by Renilla luciferase activity and normalized to the empty vector (1.0), and is shown as mean ± SEM. Experiments were performed in triplicate. Relative luciferase activity of Luciferase-Fugu parkin was normalized by Renilla luciferase activities, and the result was normalized to the empty vector control. One-way analysis of variance (ANOVA) followed by Dunnett’s post-test was used to compare the tested groups with the control. The result is shown as mean ± SEM. Experiments were performed in triplicate.

3.4.2 Effect of Nrf2 pathway activators on *parkin* transcription

3.4.2a CHO cells transfection with Fugu parkin-Luciferase construct and treated with Nrf2 pathway activators

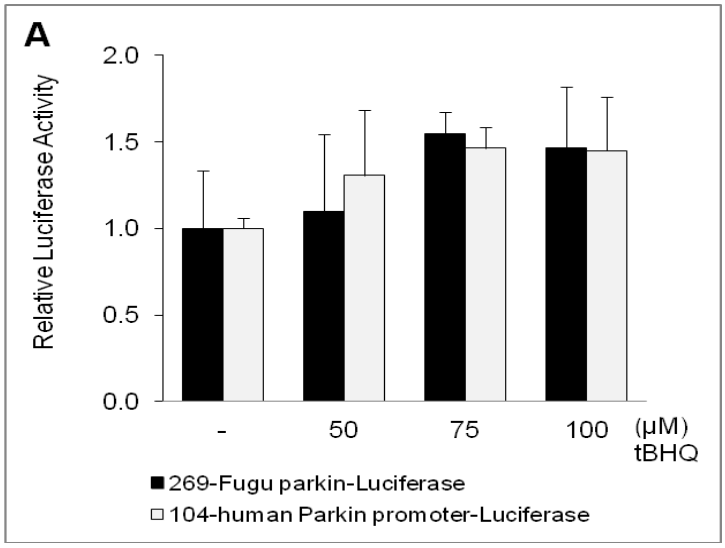
Since the Fugu *parkin* promoter region (-269 to +41) contains one potential Nrf2 site, while the human parkin promoter region (-104 to +97) does not contain a potential Nrf2 site (Asakawa et al., 2001; Yu et al., 2005; Wingender et al., 1996; Heinemeyer et al., 1998; Heinemeyer et al., 1999; Tsunoda and Takagi, 1999; Akiyama, 1995), we were interested in testing whether the potential Nrf2 site in the *parkin* promoter region was involved in *parkin* activation. Therefore, the transcriptional activity of the *parkin* promoter in the presence of Nrf2 pathway inducers was tested. CHO cells were chosen for these assays since they have been previously shown to express endogenous, functional Nrf2 that can mediate cobalt-induced HO-1 gene transcription as detected by luciferase assay (Gong et al., 2001). In CHO cells, tBHQ at concentrations from 12.5 – 100 μM oxidizes to produce H_2O_2 that induces oxidative stress and DNA damage and triggers Nrf2 activation (Phillips et al, 1989).

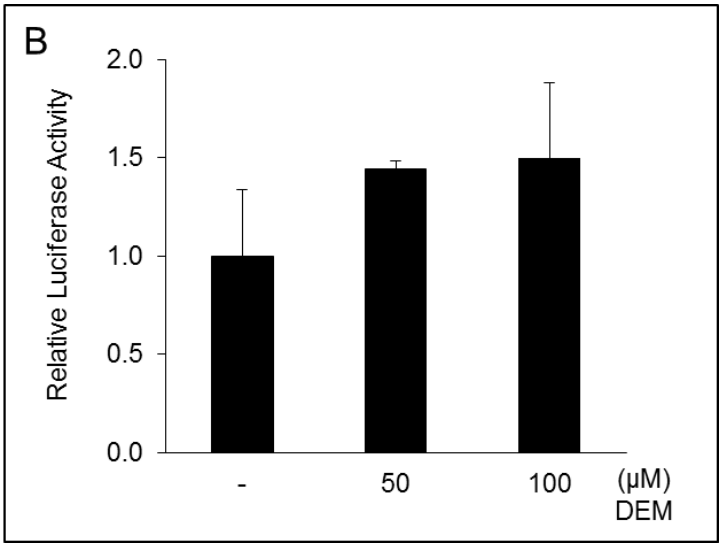
The 269-Fugu-parkin-Luciferase construct was used to moniter the effect of tBHQ on *parkin* transcription via Nrf2 pathway activation. On the other hand, the 104-human Parkin promoter-Luciferase construct served as negative control for *parkin* induction through the Nrf2 pathway since it lacks a consensus Nrf2 DNA element. Based on the above optimization studies, CHO cells were co-transfected with CMV Renilla luciferase construct (Promega) and 200 ng each of the 269-Fugu-parkin-Luciferase construct or the 104-human Parkin promoter-Luciferase construct. Transfected CHO cells were treated

with Nrf2 pathway activator tBHQ (50, 75, or 100 μM) for 24 hours. CHO cells treated with 0.05% DMSO was served as vehicle control.

As shown in Figure 15, when compared with the vehicle control, no significant difference in transcriptional activity was detected in the presence of Nrf2 pathway activator tBHQ in CHO cells transfected with either the 269-Fugu-parkin-Luciferase construct or the 104-human Parkin promoter-Luciferase construct, suggesting that the potential Nrf2 binding site located in Fugu *parkin* promoter region might not be involved in Parkin up-regulation under these experimental conditions.

In addition, CHO cells were co-transfected with the 269-Fugu-parkin-Luciferase construct and the pRL CMV Renilla luciferase construct (Promega), and then treated with Nrf2 pathway activators DEM or BSO for 24 hours. In CHO cells, treatment with DEM (100 μM) depletes glutathione levels by 99.5%, while BSO (60 μM) inhibits glutathione reductase by 64 – 114% (Freeman et al., 1985; Maran et al., 2010). The study by Saunders et al shows that depletion of glutathione (≥90%) increases HO-1 protein expression (Saunders et al., 1991). CHO cells treated with 0.05% DMSO was served as vehicle control. As shown in Figure 15, when compared with the vehicle control, no significant difference in transcriptional activity was detected in the presence of Nrf2 pathway activators DEM (B) or BSO (C), although in each case there was a trend towards an increase. These data suggested that the potential Nrf2 site located in the Fugu *parkin* promoter region might not be involved in *parkin* up-regulation under these experimental conditions.


A
Relative Luciferase Activity
2.0
1.5
1.0
0.5
0.0
-
50
75
100
(μM)
tBHQ
269-Fugu parkin-Luciferase
104-human Parkin promoter-Luciferase


B
Relative Luciferase Activity
2.0
1.5
1.0
0.5
0.0
-
50
100
(μM)
DEM

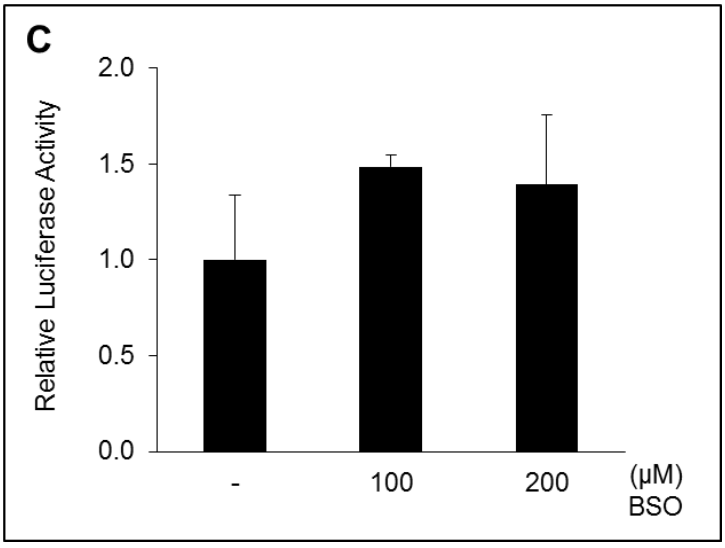

C
Relative Luciferase Activity
2.0
1.5
1.0
0.5
0.0
-
100
200
(μM)
BSO

Figure 15. Effect of Nrf2 activators on the transcription activity of Fugu *parkin* promoter. CHO cells were co-transfected with 269-Fugu parkin-Luciferase construct (200 ng) or 104-human Parkin promoter-Luciferase-pcDNA3.1(-) (200 ng) and pRL CMV Renilla luciferase construct (5 ng) (Promega). 24 hours after transfection, the cells were treated with tBHQ (50 µM, 75 µM, 100 µM) for 24 hours (A). CHO cells were co-transfected with 269-Fugu parkin-Luciferase construct (200 ng) and pRL CMV Renilla luciferase construct (5 ng). 24 hours after transfection, the cells were treated with DEM (50 µM, 100 µM) (B) or BSO (100 µM, 200 µM) (C). CHO cells treated with DMSO (0.05%) served as vehicle control. 24 hours after drug treatment, cells were lysed and luciferase activity was measured by performing the Dual-Luciferase Reporter Assay (Promega). Relative luciferase activity was normalized to *Renilla* luciferase activity, and the result was then normalized to the DMSO control. One-way analysis of variance (ANOVA) followed by Dunnett's post-test was used to compare the tested groups with the control. The result is shown as mean ± SEM. Experiments were performed in triplicate.

3.4.3 Effect of Nrf2 pathway activators on parkin protein expression

3.4.3a CHO cells treated with Nrf2 pathway activator

Besides testing whether the transcriptional activity of the Fugu *parkin* promoter increased in the presence of tBHQ, we were also interested in studying whether tBHQ could increase parkin protein levels in CHO cells. CHO cells were treated with 20 μM, 50 μM, 75 μM, or 100 μM of tBHQ; or treated with DMSO as negative control for 24 hours. As shown in Figure 16A, for Parkin protein detection, a band was observed at the 52 kDa position. However, no increase in the amount of Parkin was found in the presence of tBHQ. Proportional increase in the amount of HO-1 protein was detected with increasing concentration of tBHQ, suggesting that the tBHQ treatment has activated Nrf2 pathway.

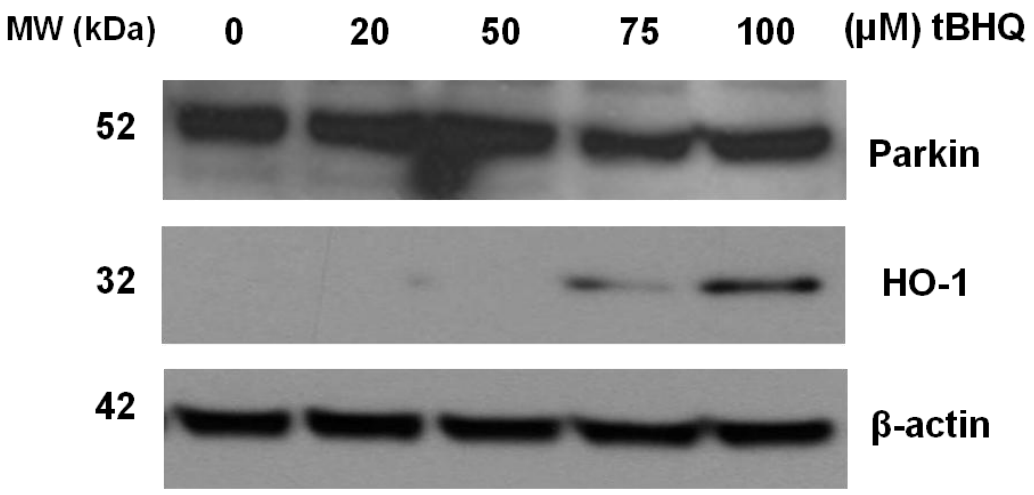


Figure 16A. CHO cells treated with Nrf2 pathway activator tBHQ. Lane 1: CHO cells were treated with DMSO (negative control); Lanes 2-5: CHO cells were treated with 20 μM, 50 μM, 75 μM, 100 μM of tBHQ. 24 hours after treatment, cells were lysed with cell lysis buffer for Western blot analysis. BCA protein assay (Thermo Scientific) was used to determine the protein concentration of cell lysates. Parkin protein was detected using anti-PARK8 mouse monoclonal antibody (1:1000) (Sigma); HO-1 protein was detected by anti-Heme Oxygenase 1 monoclonal antibody (4 μg/ml) (Clontech). β-actin protein was detected by anti-β-actin mouse monoclonal antibody (1:5000) (Abcam).

3.4.3b SH-SY5Y cells treated with Nrf2 pathway activator

In this study, we have also tested the effect of Nrf2 pathway activators *tert*-Butylhydroquinone (tBHQ), dopamine (DA), or Copper II Chloride ($CuCl_2$) on Parkin protein expression in SH-SY5Y cells. SH-SY5Y cells were treated with tBHQ (20 μM), DA (100 μM), or $CuCl_2$ (100 μM); or treated with DMSO as negative control for 6 hours. As shown in Figure 16B, when probing for Parkin protein detection by Western blotting, a band was observed at the 52 kDa position. However, no obvious increase in the amount of Parkin was found in the presence of the tested Nrf2 pathway activator. An increase in the amount of HO-1 protein was detected in cells treated with tBHQ and $CuCl_2$, suggesting that the tBHQ or $CuCl_2$ treatment has activated Nrf2 pathway in SH-SY5Y cells under these experimental conditions.

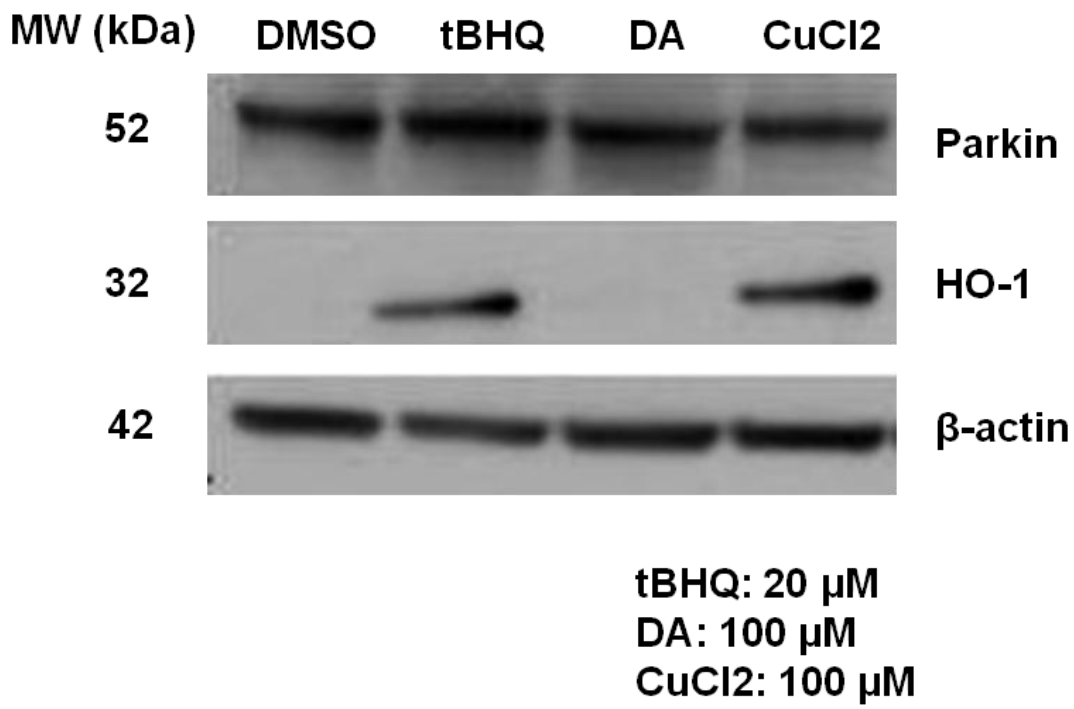


Figure 16B. SH-SY5Y cells treated with Nrf2 pathway activator. Lane 1: SH-SY5Y cells were treated with DMSO (negative control); Lanes 2-4: SH-SY5Y cells were treated with tBHQ (20 μM), DA (100 μM), or $CuCl_2$ (100 μM). 6 hours after treatment, cells were lysed with cell lysis buffer for Western blot analysis. BCA protein assay (Thermo Scientific) was used to determine the protein concentration of cell lysates. Parkin protein was detected using anti-PARK8 mouse monoclonal antibody (1:1000) (Sigma); HO-1 protein was detected by anti-Heme Oxygenase 1 monoclonal antibody (4 μg/ml) (Clontech). β-actin protein was detected by anti-β-actin mouse monoclonal antibody (1:5000) (Abcam).

3.4.3c Embryonic mouse primary neurons treated with Nrf2 pathway activator

In this study, we have also tested the effect of Nrf2 pathway activator tBHQ, and/or $CuCl_2$ on Parkin protein expression in mouse embryonic primary neurons. Since it has been shown that $CuCl_2$ could further enhance the induction effect of the tBHQ (5 μM) (Dinkova-Kostova & Wang, 2010; Wang et al., 2010), mouse embryonic primary neurons were treated with tBHQ (5 μM) in the presence or absence of $CuCl_2$; or treated with DMSO as negative control for 24 hours. As shown in Figure 16C, for Parkin protein detection, a band was observed at the 52 kDa position. However, no obvious increase in the amount of Parkin was found in the presence of the tested Nrf2 pathway activator. An increase in the amount of HO-1 protein was detected with cells treated with $CuCl_2$ (10 μM or 50 μM), or with $CuCl_2$ (50 μM or 100 μM) in the presence of tBHQ (5 μM), thus suggesting that the Nrf2 signaling pathway had been activated.

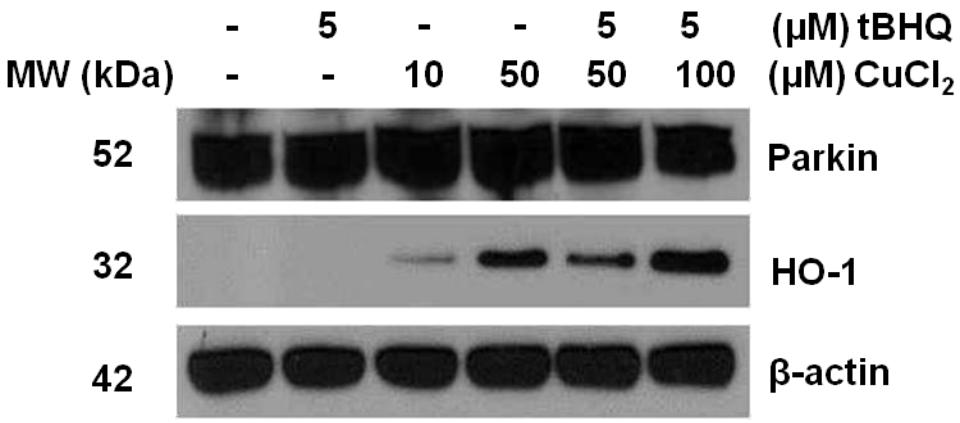


Figure 16C. Embryonic mouse primary neurons treated with Nrf2 pathway activator. Lane 1: Mouse embryonic primary neurons were treated with DMSO (negative control); Lanes 2-6: Mouse embryonic primary neurons were treated with tBHQ (5 μM), $CuCl_2$ (10 μM), $CuCl_2$ (50 μM), tBHQ (5 μM) and $CuCl_2$ (50 μM), tBHQ (5 μM) and $CuCl_2$ (100 μM), respectively. 24 hours after treatment, cells were lysed with cell lysis buffer for Western blot analysis. BCA protein assay (Thermo Scientific) was used to determine the protein concentration of cell lysates. Parkin protein was detected using anti-PARK8 mouse monoclonal antibody (1:1000) (Sigma); HO-1 protein was detected by anti-Heme Oxygenase 1 monoclonal antibody (4 μg/ml) (Clontech). β-actin protein was detected by anti-β-actin mouse monoclonal antibody (1:5000) (Abcam).

3.5 Effect of putative Nrf2 site(s) on Parkin expression

3.5.1 HEK293 cells transfection with Human Parkin promoter-Luciferase construct

Since potential Nrf2 binding sites were found in the core promoter region of human *Parkin* (Wingender et al., 1996; Heinemeyer et al., 1998; Heinemeyer et al., 1999; Tsunoda and Takagi, 1999; Akiyama 1995; Asakawa et al., 2001), we were interested in testing whether these potential Nrf2 binding sites were important for the transcriptional activity of the human *Parkin* promoter. Since HEK293 cell line is of human origin (ATCC) and expresses endogenous human Parkin (Pawlyk et al., 2003), we considered HEK293 cell line suitable for studying the effect of Nrf2 on the transcriptional activity of human *Parkin* promoter. For testing whether the potential Nrf2 binding sites located in the human *Parkin* promoter could affect the promoter's activity, HEK293 cells were transfected with 1 µg of the 104-human Parkin promoter-Luciferase construct driven by a human *Parkin* promoter containing no potential Nrf2 binding site; or with the 266-human Parkin promoter-Luciferase construct driven by a human *Parkin* promoter containing one potential Nrf2 binding site (Nrf2(1)); or with the 335-human Parkin promoter-Luciferase construct driven by a human *Parkin* promoter containing two potential Nrf2 binding sites (Nrf2(1) and Nrf2(2)). As shown in Figure 17, there was no significant difference in relative luciferase activity (transcriptional activity) among the three tested human *Parkin* promoter fragments; although the longer fragments containing the putative Nrf2 sites appeared to reduce promoter activity. These data suggested that the potential Nrf2 binding sites located in the human *Parkin* promoter might not be involved in *Parkin* activation under these experimental conditions.

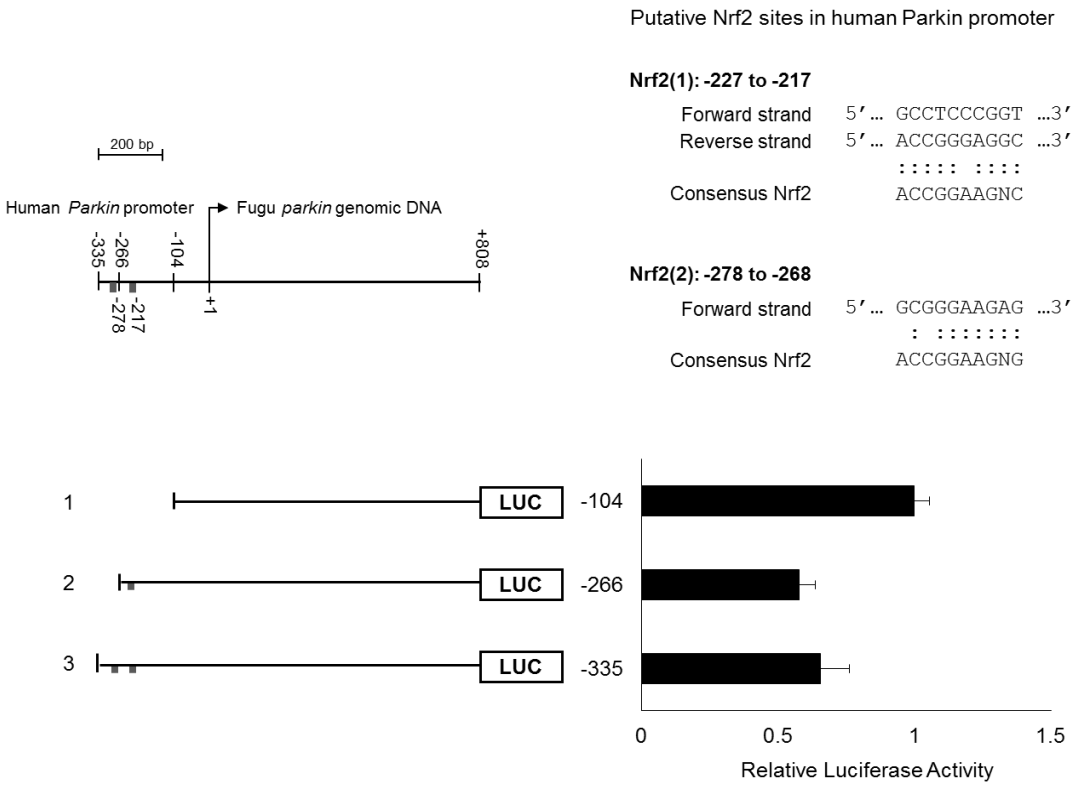


Figure 17. Effect of potential Nrf2 binding site(s) on the transcriptional activity of the human *Parkin* promoter. HEK293 cells were transfected with 1 µg of the 104-human Parkin promoter-Luciferase construct, or with the 266-human Parkin promoter-Luciferase construct, or with the 335-human Parkin promoter-Luciferase construct. For each construct, HEK293 cells were co-transfected with 5 ng of the pRL CMV Renilla luciferase construct (Promega). 36 hours after cell transfection, cells were lysed and Dual Luciferase Reporter assay (Promega) was performed to determine their luciferase activities. Relative luciferase activity of parkin-luciferase was normalized by Renilla luciferase activity, and the result was normalized to the 104-human Parkin promoter-Luciferase construct. One-way analysis of variance (ANOVA) followed by Dunnett's post-test was used to compare the tested groups with the control. The result is shown as mean ± SEM. Experiments were performed in triplicate.

3.5.2 HEK293 cells transfection with human Parkin promoter-Luciferase construct with or without potential Nrf2 binding site mutation

In order to further determine whether the potential Nrf2 binding sites are important for the transcriptional activity of the human *Parkin* promoter, site-directed mutagenesis ("*Kyle Gurley version 1.0*") (Gurley, 2004) was used to mutate the potential Nrf2 binding sites. To test the effect of Nrf2 site mutations on the transcriptional activity of human *Parkin* promoter, HEK293 cells were transfected with the 266-human Parkin promoter-Luciferase construct containing the wild-type Nrf2(1) binding sequence, or with the 266M-human Parkin promoter-Luciferase construct containing the mutated Nrf2(1) binding site. As shown in Figure 18A, there was no significant difference in transcriptional activity between the two human *Parkin* promoters with wild-type or mutated Nrf2(1) binding site, suggesting that the mutation of the potential Nrf2(1) site does not greatly influence the transcriptional activity of the human *Parkin* promoter. In addition, HEK293 cells were transfected with the 335-human Parkin promoter-Luciferase construct with the wild-type Nrf2(2) binding sequence, or the 335M-human Parkin promoter-Luciferase construct with mutated Nrf2(2) binding site. As shown in Figure 18B, there was no significant difference in transcriptional activity between the two human *Parkin* promoters with either wild-type or mutated Nrf(2) binding sites, suggesting that the mutation of the putative Nrf2(2) cis-site did not greatly influence the transcriptional activity of the human *Parkin* promoter. Interestingly, both mutants showed a trend to increased activity, perhaps indicating a weak repressor activity of the Nrf2 sites under these conditions. These results further suggested that the two potential Nrf2

binding sites are unlikely to be involved in *Parkin* gene activation under these experimental conditions.

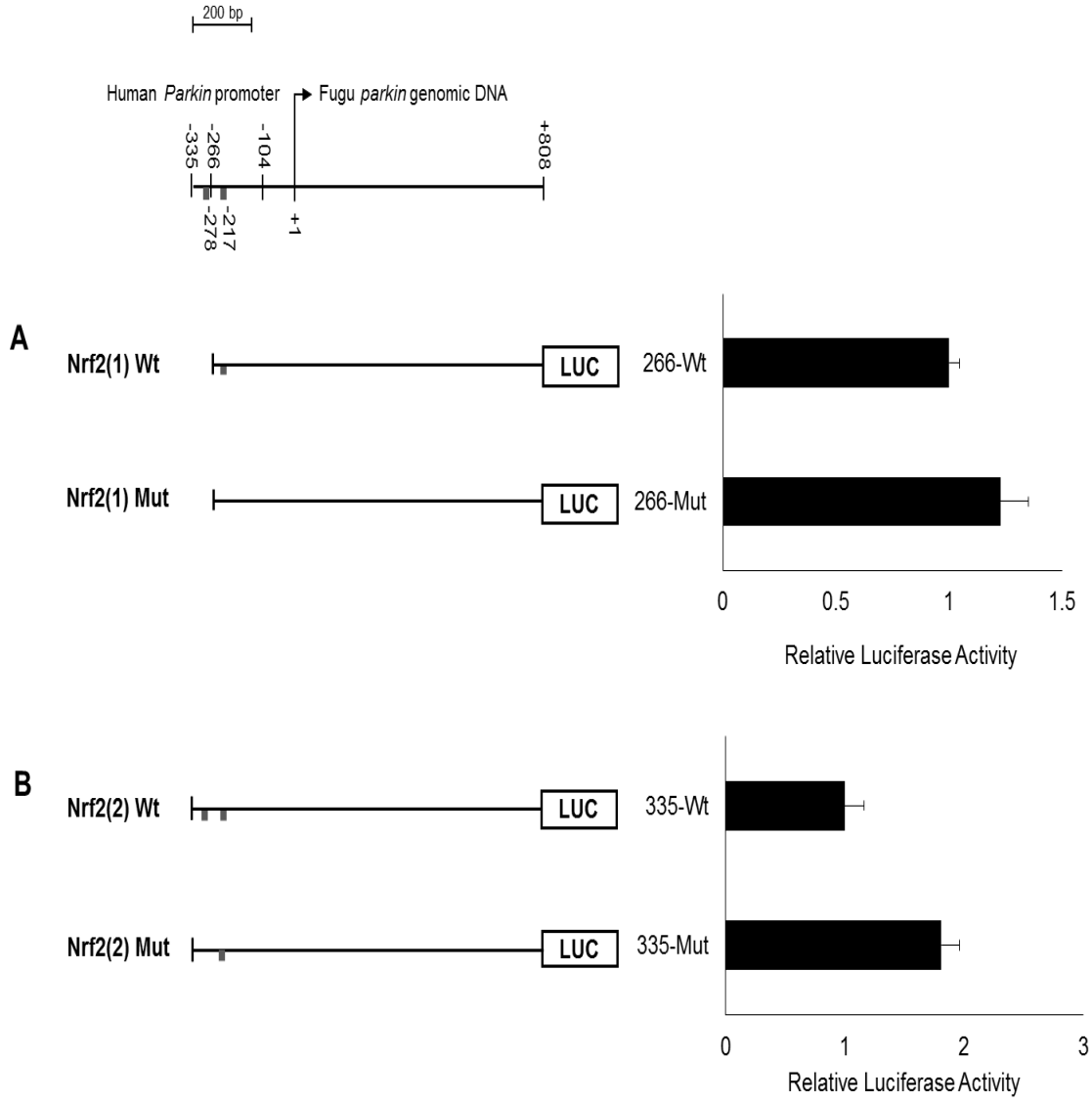

200 bp
Human Parkin promoter
Fugu parkin genomic DNA
-335
-266
-104
+808
-278
-217
+1
A
Nrf2(1) Wt
LUC
266-Wt
Nrf2(1) Mut
LUC
266-Mut
0
0.5
1
1.5
Relative Luciferase Activity
B
Nrf2(2) Wt
LUC
335-Wt
Nrf2(2) Mut
LUC
335-Mut
0
1
2
3
Relative Luciferase Activity

Figure 18. Effect of mutation of the Nrf2 binding sites on the transcription activity of human *Parkin* promoter. HEK293 cells were transfected with 1 μg of the 266-human Parkin promoter-Luciferase construct with wild-type or mutated Nrf2(1) binding sequence (A), or transfected with 1 μg of the 335-human Parkin promoter-Luciferase construct with wild-type or mutated Nrf2(2) binding sequence (B). For each construct, HEK293 cells were co-transfected with 5 ng of the pRL CMV Renilla luciferase construct (Promega). 36 hours after cell transfection, cells were lysed and Dual Luciferase Reporter assay (Promega) was performed to determine their luciferase activities. Relative luciferase activity of parkin-luciferase was normalized to Renilla luciferase activity, and the result was normalized to the Parkin promoter-Luciferase construct with wild-type Nrf2 binding site. T-test was used for comparing two groups. The result is shown as mean ± SEM. Experiments were performed in triplicate.

3.6 HEK293 cells transfection with human Nrf2 transcription factor

In order to determine if Nrf2 transcription factor is involved in regulating human *Parkin* expression, HEK293 cells (ATCC) were transfected with increasing amounts of "*pcDNA-Myc-Nrf2 plasmid*" ("Addgene Plasmid 21555") (Furukawa and Xiong, 2005) which was expected to express human Nrf2 transcription factor. Then, Nrf2, Parkin, HO-1 proteins were detected by Western blotting to see the effect of Nrf2 transcription factor on Parkin expression. Since HO-1 is a target gene of Nrf2, it served as a positive control for Nrf2 induction of its target gene (Alam et al., 1999). As shown in Figure 19, for Myc-Nrf2 protein detection using anti-Myc antibody (Cell Signaling), a single band was observed consistently at about 100 kDa position, which is greater than the predicted molecular weight of Myc-Nrf2 (~70 kDa), but consistent with endogenous Nrf2 that runs as a 100-kDa protein (Lau et al., 2013). A proportionate increase in the amount of Myc-Nrf2 protein was detected by anti-myc antibody in cells transfected with increasing amount of *"pcDNA-Myc-Nrf2 plasmid"* ("Addgene Plasmid 21555") (Furukawa and Xiong, 2005), suggesting that the Nrf2 plasmid and the cell transfection conditions were working properly. Furthermore, a proportionate increase in the amount of HO-1 was detected by anti-HO-1 antibody in cells transfected with *"pcDNA-Myc-Nrf2 plasmid"* ("Addgene Plasmid 21555") (Furukawa and Xiong, 2005), suggesting that Nrf2 transcription factor had an activation effect on its target gene in HEK293 cells. No increase in human Parkin expression was detected; on the other hand, it appeared that the expression level of Parkin was decreased, suggesting that human Parkin was not induced but possibly repressed by Nrf2 transcription factor in HEK293 cells under these experimental conditions; β-actin was detected in all lanes as a loading control.

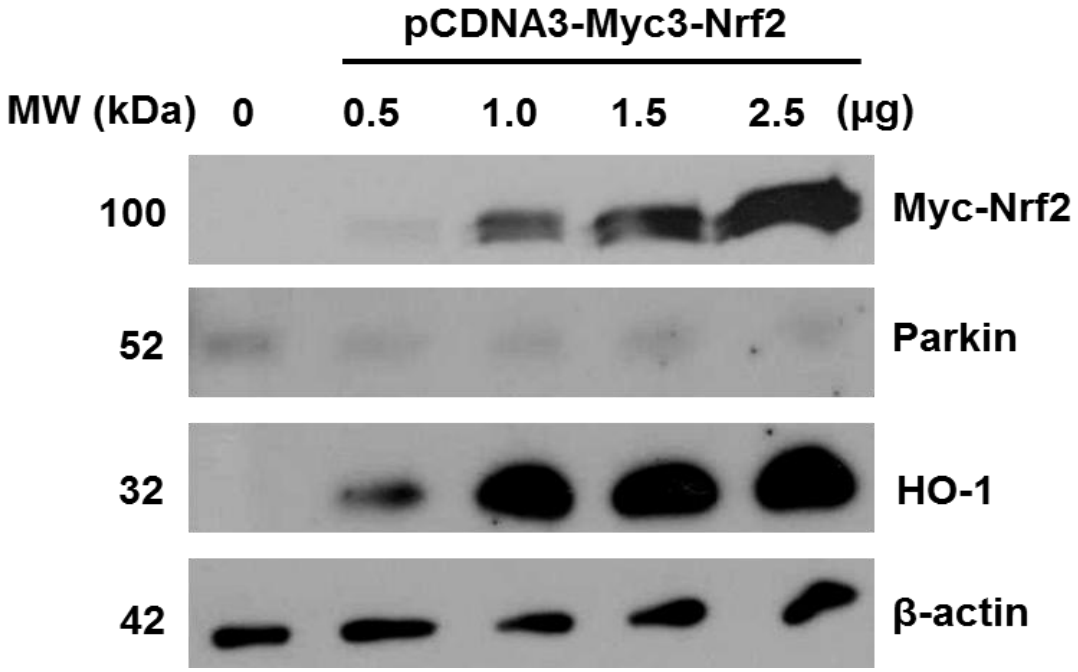
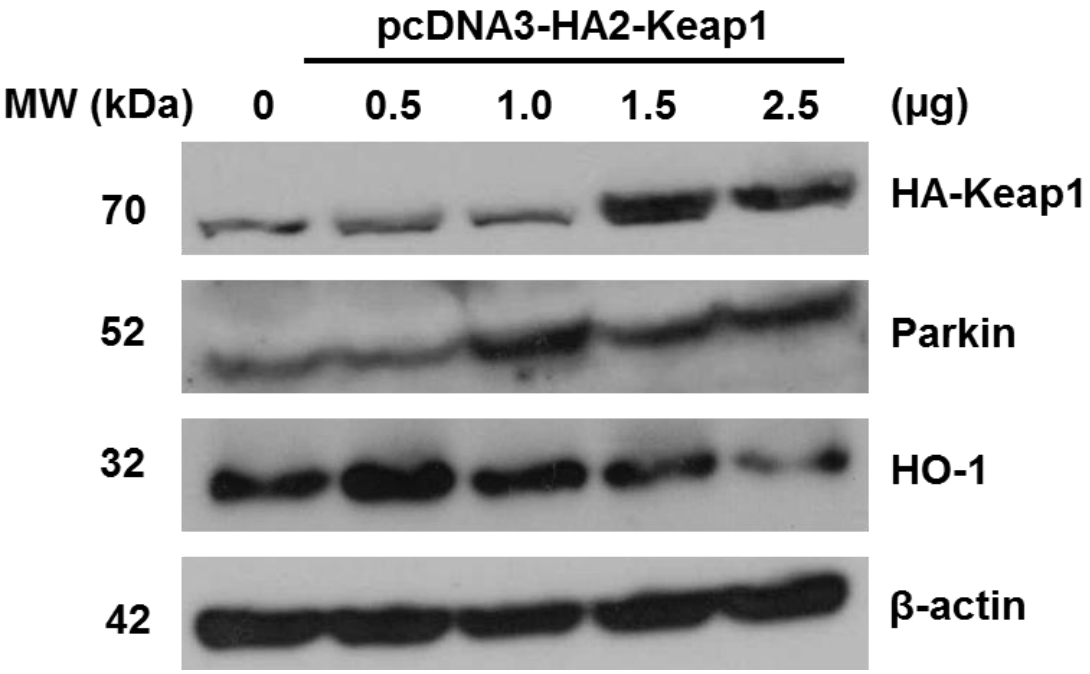

pCDNA3-Myc3-Nrf2
MW (kDa)
0
0.5
1.0
1.5
2.5
(μg)
100
Myc-Nrf2
52
Parkin
32
HO-1
42
β-actin

Figure 19. Western blot analysis of the effect of Nrf2 transcription factor on human Parkin expression. HEK293 cells (ATCC) were transfected with 0.5 μg, 1 μg, 1.5 μg, or 2.5 μg of "*pcDNA-Myc-Nrf2 plasmid*" ("Addgene Plasmid 21555") (Furukawa and Xiong, 2005). 24 hours after cell transfection, cells were lysed in cell lysis buffer for Western blot analysis. BCA protein assay (Thermo Scientific) was used to determine the protein concentration of cell lysates. Myc-Nrf2 protein was detected by anti-Myc-Tag antibody (9B11) mouse monoclonal antibody (1:1000) (Cell Signaling). Parkin protein was detected by anti-Parkin (PARK8) mouse monoclonal antibody (1:1000) (Millipore); HO-1 protein was detected by anti-Heme Oxygenase 1 monoclonal antibody (1:250) (Abcam). β-actin protein was detected by anti-β-actin mouse monoclonal antibody (1:5000) (Abcam). Horseradish Peroxidase (HRP)-Conjugated anti-mouse secondary antibody (1:10,000) was used to detect the antigen-primary antibody complex. ImageJ (National Institutes of Health) was used to measure the densitometry of protein bands; and relative amounts of Myc-Nrf2 protein, Parkin protein, HO-1 protein levels were calculated after normalization to β-actin.

3.7 HEK293 cells transfection with human Keap1

Since Keap1 is considered a repressor of Nrf2 (Baird and Dinkova-Kostova, 2011), I wanted to test whether human Parkin expression would be decreased in the presence of exogenous Keap1 in HEK293 cells. HEK293 cells (ATCC) were transfected with increasing amounts of "*pcDNA3-HA2-Keap1*" ("Addgene Plasmid 21556") (Furukawa & Xiong, 2005), which was expected to express human Keap1 protein. Then, Keap1, Parkin, HO-1 proteins were detected by Western blotting to see the effect of exogenous Keap1 protein on Parkin expression. As shown in Figure 20, about 1.2-3.5-fold increase in HA-Keap1 protein expression was detected in HEK293 cells transfected with 0.5 - 2.5 μg of "*pcDNA3-HA2-Keap1*" ("Addgene Plasmid 21556") (Furukawa and Xiong, 2005), suggesting that the plasmid and the cell transfection conditions were working properly. Since Keap1 is an inhibitor of Nrf2 (Dinkova-Kostova and Wang, 2010; Baird and Dinkova-Kostova, 2011), more Keap1 protein would repress the Nrf2 transcription factor in binding and activating its target genes, such as HO-1. A decrease in HO-1 expression was detected in HEK293 cells transfected with 2.5 μg of "*pcDNA3-HA2-Keap1*" ("Addgene Plasmid 21556") (Furukawa and Xiong, 2005). On the contrary, a slight increase (about 1.1-2.5-fold) in Parkin protein expression was detected in HEK293 cells transfected with 1.0 - 2.5 μg of "*pcDNA3-HA2-Keap1*" ("Addgene Plasmid 21556") (Furukawa and Xiong, 2005), consistent with a weak repressor activity of Nrf2 on Parkin expression. These data suggested that the Nrf2 transcription factor did not appear to be involved in the induction of Parkin but might have weakly repressed its expression, in contrast to our expectation. β-actin was detected in all lanes as loading control.



0 μg	0.5 μg	1.0 μg	1.5 μg	2.5 μg	
1.0	1.2	1.2	3.5	3.0	HA-Keap1
1.0	1.1	2.5	1.7	1.5	Parkin
1.0	1.4	1.3	1.0	0.5	HO-1

Figure 20. Western blot analysis of the effect of Keap1 on human Parkin expression. HEK293 cells (ATCC) were transfected with 0.5 μg, 1 μg, 1.5 μg, or 2.5 μg of "*pcDNA3-HA2-Keap1*" ("Addgene Plasmid 21556") (Furukawa and Xiong, 2005). 24 hours after cell transfection, the cells were lysed in cell lysis buffer for Western blot analysis. BCA protein assay (Thermo Scientific) was used to determine the protein concentration of cell lysates. HA-Keap1 protein was detected by anti-Keap1 polyclonal antibody (1:1000) (Abcam). Parkin protein was detected by anti-Parkin (PARK8) mouse monoclonal antibody (1:1000) (Millipore); HO-1 protein was detected by anti-Heme Oxygenase 1 monoclonal antibody (1:250) (Abcam). β-actin protein was detected by anti-β-actin mouse monoclonal antibody (1:5000) (Abcam). HRP-Conjugated anti-mouse secondary antibody or HRP-Conjugated anti-rabbit secondary antibody (1:10,000) was used to detect the antigen-primary antibody complex. ImageJ (National Institutes of Health) was used to measure the densitometry of the protein bands; and relative amounts of HA-Keap1 protein, Parkin protein, HO-1 protein were calculated after normalization to β-actin levels in the same sample.

CHAPTER IV

DISCUSSION

4.1 Cloning of a vertebrate construct to allow for the monitoring of an entire genomic *parkin* gene tagged with a reporter.

Since more and more people are affected with Parkinson's disease due to aging population around the world (Khandhar and Marks, 2007), it is important to have an effective therapy to prevent or slow down the process of Parkinson's disease (Singleton et al., 2013). Unfortunately, current available treatments for Parkinson's disease can only relieve symptoms, but they cannot prevent or slow down the progress of Parkinson's disease (Factor, 2008; Kalinderi et al., 2011). Moreover, the present treatments have some adverse side effects (Factor, 2008; Kalinderi et al., 2011). Therefore, there is an urgent need to discover and develop novel therapies to stop or delay the development of Parkinson's disease (Singleton et al., 2013). Although the etiology of Parkinson's disease is still unclear, it is generally accepted that the motor symptoms seen in Parkinson's disease patients are caused by the progressive degeneration and cell death of dopaminergic neurons in the substantia nigra pars compacta (Braak et al., 2003; Garrett, 2004). Human *Parkin* was considered as a new drug target for Parkinson's disease since *Parkin* mutations are associated with early-onset and late-onset Parkinson's disease (Kitada, 1998; Klein et al., 2000; Oliveira et al., 2003; Klein and Schlossmacher, 2006); and wild type Parkin protein displays a number of cell protective effects (Ulusoy and Kirik, 2008). Therefore, increasing the level of wild-type Parkin might be able to rescue the cells in danger and prevent cell death in the substantia nigra (Ulusoy and Kirik, 2008). To increase *Parkin* expression, it is important to identify the cis- and trans-acting transcriptional regulatory factors and signaling pathway involved in its expression (Maston et al., 2006). However, determining the transcription elements and pathways of

human *Parkin* has been difficult because of its large size (Kitada et al., 1998; Asakawa et al., 2001; Yu et al., 2005). Human *Parkin* gene contains large introns, and the entire gene is about 1.4 Mb (Megabase) (Kitada et al., 1998; Asakawa et al., 2001) (Figure 1). Fortunately, Yu *et al.* described an ortholog of human *Parkin* in Fugu fish (Fugu *parkin* gene) (Yu et al., 2005). Since Fugu *parkin* gene has much smaller sized introns than the human *Parkin*, so Fugu *parkin* is about 350-fold smaller than human *Parkin* (Figure 1) (Yu et al., 2005). Despite of its small size, Fugu *parkin* is similar to the human *Parkin* in terms of "genomic structure", gene function and "tissue expression pattern" (Yu et al., 2005). Therefore, Fugu *parkin* gene might be a suitable model for studying the transcription factors and signaling pathways involved in *Parkin* expression (Yu et al., 2005).

Hence, in this study, we have used the Fugu *parkin* genomic clone (Yu et al., 2005) as a model to study the cis- and trans- transcriptional factors and signaling pathway involved in *parkin* expression. Based on the Fugu parkin-eGFP-pDrive construct (Figure 5) (May-McNally et al., 2010), we have successfully cloned several Fugu parkin-eGFP constructs (Figure 6), Fugu parkin-Luciferase constructs (Figure 7), human Parkin-promoter-Luciferase constructs (Figure 8), and human Parkin-promoter-Luciferase constructs with mutations in the putative Nrf2 sites (Figure 9) for this study. After testing the expression of the Fugu parkin-eGFP constructs by using fluorescence microscopy and Western blotting (Figure 11), I found that the 5'-flanking region of Fugu *parkin* might contain repressive element hindering the expression of Fugu *parkin* since no Fugu *parkin* expression was detected in cells transfected with Fugu parkin-eGFP constructs with full 5'-flanking region of Fugu *parkin* despite the presence of CMV promoter (Figure 11),

whereas Fugu parkin-eGFP expression was detected in cells transfected with Fugu parkin-eGFP constructs lacking 5'-flanking region of Fugu *parkin* (Figure 11). Moreover, the result of dual luciferase assay suggested that the presence of the entire 5'-flanking region of Fugu *parkin* might repress the transcriptional activity of Fugu *parkin* (Figure 12). Furthermore, our experimental results suggested that an enhancer region extending from -269 to -100 bp might be important for the transcription activity of Fugu *parkin* (Figure 13). Not only could the Fugu *parkin* driven by its own promoter sequence, but it could be driven by the human *Parkin* promoter sequence (Figures 14A & 14B). The activity of both 269-Fugu and 104-human Parkin promoters were similar although fairly weak, inducing only around 5-fold enhancement of lucifierase activity compared to vector. However, similar weak promoter activity has been observed for other neuronal genes, such as the dopamine-D2 receptor promoter (Arinami et al. 1997). Therefore, these Fugu parkin reporter constructs could be used for studying the potential cis-acting transcriptional elements found in the *parkin* gene and also the candidate transcriptional factors. Moreover, the changes in *parkin* expression could be monitored conveniently since the *parkin* gene was tagged with eGFP or luciferase as reporter.

4.2 No correlation between the presence of Nrf2 binding site in *parkin* promoter and the up-regulation of *parkin* was found in this study

According to the "*TRANSFAC*" transcription factor database, "*TFBIND*" and "*TFSEARCH*" softwares, the promoter region of Fugu *parkin* (-269 to +41) contains one potential Nrf2 binding site (Figure 3) (Yu et al., 2005; Wingender et al., 1996;

Heinemeyer et al., 1998; Heinemeyer et al., 1999; Tsunoda and Takagi, 1999; Akiyama 1995). Our experimental results showed that Nrf2 pathway activators including tBHQ, DEM, and BSO did not appear to increase the transcriptional activity of the Fugu *parkin* promoter under the specific experimental conditions in this study (Figure 15), so we could not find an association between the presence of Nrf2 binding site in *parkin* promoter region with *parkin* up-regulation in this study. Importantly, these activators did increase HO-1 protein levels indicating activation of Nrf2 signaling (Figure 16). Moreover, I tested whether the two identified putative Nrf2 binding sites were important for transcriptional activation of human *Parkin* promoter by comparing the transcriptional activity of three human *Parkin* promoter fragments with or without the potential Nrf2 binding sites. However, no significant difference in transcriptional activity was found among the three tested human *Parkin* promoter fragments (Figure 17) and no significant difference in transcriptional activity was found between the human *Parkin* promoters containing wild-type or mutated Nrf2 binding site(s) under the specific experimental conditions (Figure 18). Thus, in contrast to our expectation we could not find a link between the presence of Nrf2 binding sites in human *Parkin* promoter and the up-regulation of human *Parkin* in this study. A previous study has shown that functional Nrf2 expressing in CHO cells could activate HO-1 transcription (Gong et al., 2001), so we also included detection of HO-1 in our experiment as a positive control for the Nrf2 pathway activation. More experiments have to be done in order to further study the relationship between Nrf2 transcription factor activity and vertebrate *parkin* gene activation.

4.3 No correlation between Nrf2 pathway activaton and human Parkin protein induction was found in this study

In this study, we could not find an obvious increase in parkin protein expression level in the presence of any of the Nrf2 activators tested in CHO cells, SH-SY5Y cells, or embryonic mouse primary neurons, while HO-1 induction suggested that the Nrf2 pathway was activated in these cells (Figure 16). A proportionate increase in the expression levels of Myc-Nrf2 protein was detected in HEK293 cells transfected with increasing amount of "*pcDNA-Myc-Nrf2*" plasmid ("*Addgene Plasmid 21555*") (Furukawa and Xiong, 2005) (Figure 19), suggesting that the Nrf2 plasmid and cell transfection were working properly. Besides, induction of HO-1 suggested that exogenous Nrf2 transcription factor was functional and could activate its target gene such as HO-1 in HEK239 cells (Figure 19). Therefore, if *Parkin* were a target gene of Nrf2 activity, the expression level of Parkin protein would increase in the presence of exogenous Nrf2 transcription factor. However, we could not detect an obvious increase in human Parkin protein expression level under the specific experimental conditions employed (i.e., Western blotting) (Figure 19).

An increase in Keap1 protein expression level was detected in HEK293 cells transfected with increasing amount of "*pcDNA3-HA2-Keap1*" ("*Addgene Plasmid 21556*") (Furukawa and Xiong, 2005), suggesting that the Keap 1 plasmid and the cell transfection were working properly (Figure 20). Since HO-1 was a target gene of Nrf2 (Alam et al., 1999), and Keap1 could function as a repressor of Nrf2 (Dinkova-Kostova and Wang, 2010; Baird and Dinkova-Kostova, 2011); so higher Keap1 expression level would inhibit more Nrf2 transcription factor in binding and activating its target gene such as

HO-1. Therefore, HO-1 expression was expected to decrease with increasing expression level of Keap1. As expected, there was a decrease in HO-1 protein expression level in the presence of larger amount of Keap1 protein, implying that Nrf2 transcription factor was inhibited by Keap1 from activating its target gene HO-1 in HEK293 cells (Figure 20). Therefore, if human *Parkin* were a target gene of Nrf2 transcription factor, a decrease in human Parkin protein level should be detected with increasing amounts of Keap1 protein. However, we could not detect an obvious decrease in human Parkin protein expression level under the specific experimental condtions in this study (Figure 20). Consistent with a potential repressor function of Nrf2 on *Parkin* transcription, Keap1 actually appeared to potentially increase Parkin protein levels (Figure 20), however, since I had difficulties in obtaining a specific and consistent anti-Parkin antibody for this study, further experiments have to be carried out to confirm if such increase is statistically significant. Since the up-regulation of Parkin might be cell specific, we could also test it in other cell lines (beyond the ones used in this study).

4.4 Conclusion and Future Directions

In this study, we have cloned various Fugu parkin reporter constructs in which genomic Fugu *parkin* gene was tagged with a reporter (eGFP or luciferase) for studying the cis- and trans- transcriptional factor and signaling pathway involved in *parkin* gene expression. Our experimental results suggest that there might be an upstream repressor in the 5'-flanking region of Fugu *parkin* that inhibits its expression. Fugu *parkin* gene could be driven by its own promoter sequence or human *Parkin* promoter, thus allowing us to

study the potential cis- and trans- transcription factors involved in *Parkin* expression. The promoter region of Fugu *parkin* (-269 to +41) contained one potential Nrf2 binding site, while the promoter region of human *Parkin* (-335 to +97) had two potential Nrf2 binding sites (Asakawa et al., 2001; Yu et al., 2005; Wingender et al., 1996; Heinemeyer et al., 1998; Heinemeyer et al., 1999; Tsunoda and Takagi, 1999; Akiyama, 1995). Based on our experimental results, we could not find that these potential Nrf2 binding sites located at the Fugu *parkin* promoter and human *Parkin* promoter were involved in *parkin* activation. However, there are a number of potential Nrf2 binding sites locate in intron 1 of human *Parkin* which might be important for *Parkin* expression (Asakawa et al., 2001; Wingender et al., 1996; Heinemeyer et al., 1998; Heinemeyer et al., 1999; Tsunoda and Takagi, 1999; Akiyama, 1995), so in order to facilitate the screening of the effects of these potential Nrf2 binding sequences, Chromatin immunoprecipitation assay (ChIP) or electrophoretic mobility shift assay (EMSA) might be good approaches to detect interactions between Nrf2 transcription factor and the potential Nrf2 binding sequences found in *parkin* gene. However, the results revealed that Nrf2 activators or transfection of Nrf2 were ineffective to induce Parkin protein levels, even though HO-1 protein was induced. Conversely, inhibition of endogenous Nrf2 by transfection of Keap1 failed to reduced Parkin protein levels even though HO-1 levels were reduced. Taken together, these findings suggested that Nrf2 has little -if any- role in the regulation of *Parkin* expression, as least in the cell lines that we tested under the specific experimental conditions.

There are several limitations of this study, in which the biggest challenge is the detection of endogenous Nrf2 protein. We have problems in detecting the endogenous Nrf2 protein

by Western blotting. It is important to mention that, as of today, the migration pattern of Nrf2 protein (observed molecular weight) detected by Western blotting is still controversial (Narasimhan et al., 2012; Lau *et al*., 2013). It has been reported that the predicated molecular weight of human Nrf2 protein was approximately 66 kDa (Moi *et al.*, 1994). However, a number of studies had detected bands at various sizes ranging from ~57-110 kDa in Nrf2 detection by Western blotting (Pi et al., 2007; Leiser & Miller, 2009; Kapeta et al., 2010; Narasimhan et al., 2011; Kim et al., 2012; Narasimhan et al., 2012). Moreover, previous studies suggested that the ~95–110 kDa bands in Nrf2 detection might be due to post-translational modification of Nrf2 such as phosphorylation and polyubiquitination**,** or because of "*high acidic charges*" within Nrf2, or be the result of SDS-stable "*dimer formation*" (Moi et al., 1994; Kang et al., 2002; Li et al., 2005; Narasimhan et al., 2012). Therefore, the higher molecular weight of Nrf2 protein detected here might be due to one or more reasons mentioned above. An alternate explanation might be the selected activators used in this study for increasing Nrf2 function by allowing Nrf2 translocation from cytoplasm to nucleus to induce its target gene under the specific experimental conditions, rather than increasing the Nrf2 expression level, so we could not detect a proportional increase in endogenous Nrf2 protein expression level. If this were the case, HO-1 protein expression level could serve as an indirect indicator of Nrf2 pathway activation in Western blot analysis. We could also use other methods to detect Nrf2 pathway activation. For example, if we could perform cell fractionation to separate the cell lysate into cytoplasm and nucleus, we might have a better chance to determine if Nrf2 translocated from cytoplasm to nucleus in the presence of Nrf2 pathway activators. Alternately, we could use immunostaining to detect the localization

of Nrf2 (in cytoplasm or in nucleus). In addition, for testing whether the pupative Nrf2 binding sites are inovled in the regulation of *parkin* gene, cells transfected with the Fugu-parkin luciferase constructs should be treated with Nrf2 pathway activator(s); and a positive control (e.g. HO-1 vector) should be included in the dual lucifease assay in order to confirm that Nrf2 pathway is activated and Nrf2 can up-regulate its target gene in the cell lines tested under the specific experimental conditions. These have to be done in future experiments. Moreover, in Western blot analysis of Parkin protein expression, in order to confirm that the bands detected are Parkin (instead of non-specific bands), we should include a positive control (e.g. cells transfected with a human Parkin cDNA). Moreover, in order to compare the expression level of Parkin protein detected by Western blotting, we should avoid over-exposure or over-loading the gel with Parkin protein. These have to be done in future experiments.

It is also worthy to mention that I had difficulties in mutating the putative Nrf2 sites initially and had spent months with troubleshooting. Finally, I had used another site-directed mutagenesis protocol ("*Kyle Gurley version 1.0*") (Gurley, 2004) and mutated the putative Nrf2 sites successfully which were verified by DNA sequencing. Based on this experience, I suggest using this method for site-directed mutagenesis in future studies.

In addition to these experiments, I have modified and optimized the steps for preparing cleaner MBP-Parkin proteins and sent them to our collaborator for studying the enzymatic activity of Parkin protein (Appendix 1). In addition, I have cloned Histidine-tagged human Parkin construct for the enzymatic activity study (Appendix 2). Moreover, for the project of studying and comparing the levels and activity of mitochondria creatine kinase (mtCK) and aconitase (Aco2) in *parkin* knockout mice and control wild type mice,

I have isolated mitochondria from mouse brains and checked the purity of the isolated mitochondria with various marker proteins (Appendix 3). These collective experiments have informed Parkin protein function-related ongoing studies by my colleagues in the laboratory.

Appendices

1. Preparation and purification of MBP-Parkin protein

In order to further study the post-translational modification of human Parkin, I have prepared the Maltose-Binding-Protein (MBP)-Parkin recombinant proteins. Constructs of MBP-Parkin cDNAs of wild type (full length Parkin) and various deletions clones (IBR-RING2, 311-stop, 416-stop) in the PMAL-p2T were given by Dr. Keiji Tanaka's laboratory (Matsuda et al., 2006). The full length Parkin contains all the 465 amino acids; while the 311-stop mutant Parkin contains only 311 amino acids and stops at the IBR region; while the 416-stop mutant Parkin contains only 416 amino acids and stops at the end of IBR region; while the IBR-RING mutant contains only the IBR-RING2 region (295 to 465 amino acids) of human Parkin protein (Matsuda et al., 2006).

MBP-Parkin preparation and purification was carried out according to the procedures suggested by the manufacture (New England Biolabs), and the method published in previous paper (Matsuda et al., 2006), and protocol provided by Dr. Qiubo Jiang in Dr. Michael Schlossmacher's laboratory. Briefly, MBP-Parkin was expressed in modified E.coli strain BL21(DE3) condon-plus (RIL) strain (Statagene) (Matsuda et al., 2006) according to the manufacturer's instruction, then MBP-Parkin was purified from bacteria lysate using gravity-flow chromatography column (Bio-Rad) with amylose beads (New England BioLabs) according to the manufacturers' instructions, and suitable column buffer ("*20 mM Tris-HCL, pH 7.5, 200 mM NaCl, 1mM dithiothreitol, and 100 μM ZnSO4*") (Matsuda et al., 2006). Finally protein was eluted by 10 mM maltose (Matsuda et al., 2006) (New England Biolabs). Finally, silver staining (Sigma-Aldrich) was

performed according to the procedures recommended by the manufacture to check the eluted Pakrin protein (Figure 21).

When I prepared and purified the MBP-Parkin using the suggested protocols, the purity of the MBP-proteins was not satisfactory. Since the purity of protein is crucial for the enzyme activity assay, so I had tried different experimental conditions to find out how to improve the purity. Finally, I found that by using more selective gradient elution, the purity of the MBP-Parkin was improved. The purified MBP-Parkin proteins have been sent to our collaborator Dr. Rajib Sengupta (Karolinska Institutet) for enzymatic activity experiments.

2. Cloning of Histidine-Parkin construct

I have cloned the Histidine-tagged human Parkin construct for enzymatic activity study of human Parkin. The cloning involved multiple steps. In short, I had excised the human Parkin cDNA (1397 bp) from the MBP-tagged human Parkin construct (wild type full length Parkin protien) given by Dr. Keiji Tanaka (Matsuda et al., 2006) by enzymatic digestion using the *BamH*I enzyme (New England Biolabs). Then, the full length human *Parkin* cDNA sequence was inserted into pcDNA3.1-His A plasmid (Invitrogen). Then, the Histidine-human Parkin DNA sequence was amplified by PCR. The PCR product was extracted from agarose gel and DNA elution was performed by using NucleoSpin® Extract II according to the manufacturer's recommended procedures (MACHEREY-NAGEL). Then, the sequence of Histidine-human Parkin DNA was inserted into a bacteria expression vector pMAL-c2.

Figure 21.

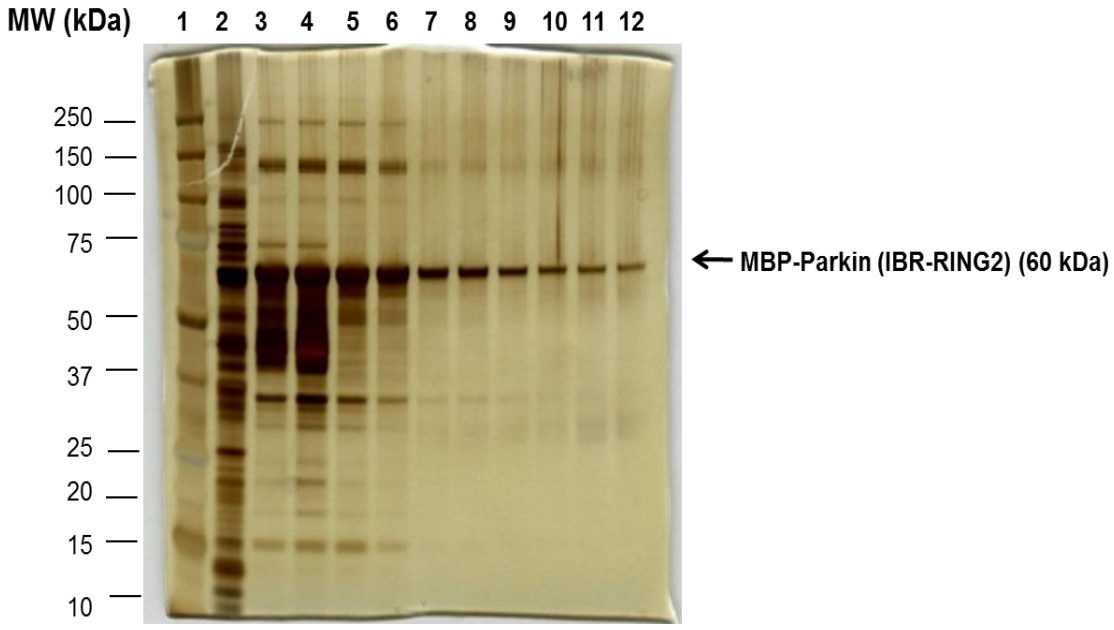


Figure 21. Representative image of silver staining of eluted MBP-Parkin protein. Lane 1: Protein marker (Bio-Rad); lane 2: Input MBP-Parkin protein before passing through gravity-flow chromatography column (Bio-Rad); lanes 3-12: Eluted MBP-Parkin protein with 0.5 µM (micromolar), 1 µM, 2 µM, 3 µM, 4 µM, 5 µM, 6 µM, 8 µM, 9 µM, 10 µM maltose, respectively.

3. Isolation and purification of mitochondria

The procedures for isolating and purifying mitochondria were performed according to the protocol provided by Heather Boston in Dr. Michael Schlossmacher's laboratory with some modifications: mice brains were homogenized and then centrifuged at 4 °C to separate into three fractions: a) cytosolic; b) endoplasmic reticulum (ER) and Golgi; c) mitochondria and lysosome. The purity of mitochondria was checked by using various mitochondria markers, ER markers, and lysosome markers by Western blot analysis. Mitochondria markers: Aconitase (ACO2) (Beinert and Kennedy, 1993), Mitochondria Creatine kinase (MtCK) (Wood et al., 1995), Translocase of outer membrane (Tom20) (Saitoh et al., 2007), Voltage-dependent anion channel (VDAC) (Hoogenboom et al., 2007). ER markers: Glucose-regulated protein 78 (GRP78) (Hendershot et al., 1988). Lysosome markers: Cathepsin D (Faust et al., 1985), β-glucosidase (GBA) (Erickson et al., 1985).

Aconitase (ACO2): Aconitase is a 83 kDa protein expresses in mitochondria (Beinert and Kennedy, 1993). Besides, aconitase was also found in cytoplasm (Beinert and Kennedy, 1993). In this study, aconitase was detected by anti-Aconitase rabbit polyclonal antibody (ProteinTech Group).

Mitochondria creatine kinase is a 43 kDa enzyme localizes in the mitochondria (Wood et al., 1995). In this study, mitochondria creatine kinase was detected by anti-creatine kinase MT mouse monoclonal antibody (Abcam).

Translocase of outer membrane (Tom20) is a 20 kDa protein localizes in mitochondria (Saitoh et al., 2007). In this study, Tom20 protein was detected by the anti-Tom20 mouse monoclonal antibody (BD Transduction Laboratories).

Voltage-dependent anion channel (VDAC) is a 30 kDa protein localizes in the mitochondria (Hoogenboom et al., 2007). In this study, VDAC protein was detected by anti-VDAC mouse monoclonal antibody (MitoSciences).

Glucose-regulated protein 78 (GRP78) is a protein generally localizes in the endoplasmic reticulum; and its molecular weight is 78 kDa (Hendershot et al., 1988). However, previous study found that, in stressful condition, GRP78 tented to localize in the mitochondria (Sun et al., 2006). In this study, GRP78 was detected by anti-GRP78 mouse monoclonal antibody (BD Transduction Laboratories).

Cathepsin D is an enzyme localizes in the lysosome (Faust et al., 1985). There are various forms of Cathepsin: preprocathepsin D (43 kDa), procathepsin D (46 kDa), cathepsin D heavy chain (30 kDa), and cathepsin D light chain (15 kDa) (Erickson et al., 1981). The cathepsin D heavy chain and cathepsin D light chain are the enzymatically active forms of cathepsin D (Erickson et al., 1981). In this study, cathepsin D was detected by anti-Cathepsin D rabbit polyclonal antibody (Cell Signaling).

β-glucosidase (GBA) is an enzyme localizes in the lysosome; and the mature form of GBA is about 60 kDa (Erickson et al., 1985). In this study, GBA protein was detected anti-GBA rabbit polyclonal antibody (Santa Cruz).

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